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(57) Abstract

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is idependently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

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AGONIST PEPTIDE DIMERS

FIELD OF THE INVENTION

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO) is a glycoprotein horm ne with an approximate molecular weight of 34,000 daltons. The primary role of EPO, which is synthesized in the kidneys of mammals, is to stimulate mitotic cell division and differentiation of erythrocyte precursor cells. As a result, EPO acts to stimulate and to

regulate the production of erythrocytes. Erythrocytes, and the hemoglobin contained therein, play a central role in supplying oxygen to the body. Thus, the stimulation of erythrocyte production is able to increase the oxygen-carrying capacity of the blood.

During normal conditions, EPO is present in very low concentrations in plasma. Under hypoxic conditions, the amount of EPO in the circulation is increased in response to reduced O_2 blood levels. Hypoxia may be caused from various conditions including the loss of large amounts of blood, destruction of red blood cells by over-exposure to radiation or chemotherapeutic agents, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or by various forms of anemia. As the hypoxic condition diminishes, the amount of EPO produced subsequently diminishes.

Because of the essential role of EPO in red blood cell formation, the hormone is useful in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. Recent studies provide a basis for the efficacy of EPO therapy in a variety of disease states, disorders, and states of hematologic irregularity, including: beta-thalassemia (See, Vedovato et al. (1984) Acta. Haematol. 71:211-213); cystic fibrosis (See, Vichinsky et al. (1984) J. Pediatric 105:15-21); pregnancy and menstrual disorders (See, Cotes et al. (1983) Brit. J. Ostet. Gyneacol. 90:304-311); early anemia of prematurity (See, Haga et al. (1983) Acta Pediatr. Scand. 72:827-831); spinal cord injury (See,

Claus-Walker et al. (1984) Arch. Phys. Med. Rehabil. 65:370-374); space flight (See, Dunn et al. (1984) Eur. J. Appl. Physiol. 52:178-182); acute blood loss (See, Miller et al. (1982) Brit. J. Haematol. 52:545-590); aging (See, Udupa et al. (1984) J. Lab. Clin. Med. 103:574-588); various neoplastic disease states accompanied by abnormal erythropoiesis (See, Dainiak et al. (1983) Cancer 5:1101-1106); and renal insufficiency (See, Eschbach et al. (1987) N. Eng. J. Med. 316:73-78).

Although purified, homogenous EPO has been characterized, little is known about the mechanism of EPO-induced erythroblast proliferation and differentiation. The specific interaction of EPO with progenitor cells of immature red blood cells, platelets, and megakaryocytes has not been described. This is due in part, to the small number of surface EPO receptor molecules on normal erythroblasts and on the erythroleukemia cell lines. See Krantz and Goldwasser (1984) Proc. Natl. Acad. Sci. USA, 81:7574-7578; Branch et al. (1987) Blood 69:1782-1785; Mayeux et al. (1987) FEBS Letters 211:229-223; Mufson and Gesner (1987) Blood 69:1485-1490; Sakaguchi et al. (1987) Biochem. Biophys. Res. Commun. 146:7-12; Sawyer et al. (1987) Proc. Natl. Acad. Sci. USA 84:3690-3694; Sawyer et al. (1987) J. Biol. Chem. 262:5554-5562; and Todokoro et al. (1988) Proc. Natl. Acad. Sci. USA 84:4126-4130. The DNA sequences and encoded peptide sequences for murine and human EPO receptor proteins have been described. See, D'Andrea et al. PCT Patent Publication No. WO 90/08822 (published 1990).

The EPO-receptor (EPO-R) belongs to the class of growth-factor-type receptors which are activated by a ligand-induced protein dimerization. Other hormones and cytokines such as human growth hormone (hGH), granulocyte colony stimulating factor (G-CSF), epidermal growth factor (EGF) and insulin can cross-link two receptors resulting in juxtaposition of two cytoplasmic tails. Many of these dimerization-activated receptors have protein kinase domains within the cytoplasmic tails that phosphorylate the neighboring tail upon dimerization. While some cytoplasmic tails lack intrinsic kinase activity, these function by association with protein kinases. The EPO receptor is of the latter type. In each case, phosphorylation results in the activation of a signaling pathway.

In accordance with the present invention, it has been discovered that the dimerization of peptide agonists and antagonists of dimerization-mediated receptors, such as EPO-R, increase the biological efficacy relative to the biological activity of the 'monomeric' agonists and alters the properties of the antagonists such that, these dimers function as agonists, exhibiting biological activity.

SUMMARY OF THE INVENTION

In a first embodiment, the present invention is directed to peptide dimers which behave as cell-surface receptor agonists, dimers which exhibit binding and signal initiation of growth factor-type receptors. In one embodiment, the present invention provides peptide dimers which behave as EPO agonists. These

dimers have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 residues in length, comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can b C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ~~ID~~ NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

More preferably, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3), where each amino acid is indicated by standard one letter abbreviation; each X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is

homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

In a more preferred embodiment, both X_3 and X_8 are C and thus, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 4). More preferably, the monomeric peptide unit comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 5), where X_4 can be R or H; X_5 can be F or M; X_6 can be I, L, T, M, or V; X_7 is D or V; X_9 can be G, K, L, Q, R, S, or T; and X_{10} can be A, G, P, R, or Y. In a most preferred embodiment, the monomeric peptide unit of the dimer will comprise a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 6), where X_1 can be D, E, L, N, S, T, or V; X_2 can be A, H, K, L, M, S, or T; X_4 is R or H; X_9 can be K, R, S, or T; and X_{10} is P. Particularly preferred monomeric peptide units of the dimers include:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and

SCHFGPLTWVCK (SEQ ID NO: 18).

Other particularly preferred monomeric peptide units of the present dimers include peptides comprising a core sequence of the formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When (AX_2) is present, i.e. when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to delete the Y, tyrosine residue in SEQ ID NOS. 21 - 93. Such monomeric peptides can also be prepared by substitution of Y in position A in the peptides of Fig. 9.

In accordance with the present invention the monomeric units of the dimers can be the same or different.

In a preferred embodiment polyethylene glycol (PEG) is employed as a linker to form the dimeric peptides of the present invention through a covalent bond.

In another embodiment, the present invention is directed to pharmaceutical compositions comprising at least one dimer peptide of the invention and a pharmaceutical carrier.

In a further embodiment, the present invention provides a method for therapeutically treating a mammal having a condition resulting from a hormone or growth factor deficiency by administration of at least one of the dimer peptides of the present invention.

In a still further embodiment, a method for therapeutically treating a mammal having a condition resulting from a deficiency of EPO or from reduced levels of blood oxygen caused by a decrease in erythrocyte number is provided.

In another embodiment of this invention, a method is provided for preparing agonists of cell-surface receptors wherein agonists of the class of cell-surface or dimerization-mediated receptors are dimerized to enhance the in vitro or in vivo biological activity of the cell-surface receptor relative to the monomeric agonists from which the dimer is derived. This method is also directed to the preparation of agonists of such growth-factor-type receptors by dimerizing antagonists of these receptors; the dimerized 'antagonists' thereby exhibit agonist biological activity in vitro and in vivo. In a preferred embodiment, the present method is directed to the preparation of EPO-R dimer agonists from monomeric EPO-R antagonists.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a major peak, with a retention time of 37 minutes, of the dimerized EPO peptide, GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8)

Fig. 2 shows a major peak, with a retention time of 48 minutes, following purification of the dimerized EPO peptide, (SEQ ID NO: 8).

Fig. 3 depicts the MALDI-TOF mass spectral analysis of the dimerized peptides, including peptide (SEQ ID NO: 8), GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13) and SCHFGPLTWVCK (SEQ ID NO: 18).

Fig. 4 shows the SDS-PAGE analysis of DPDPB crosslinking of EPO binding protein (EBP) in the presence and absence of EPO agonist peptides.

Fig. 5 demonstrates equilibrium EPO binding to immobilized EPO binding protein. Panel A represents the equilibrium binding data and Panel B (inset) is the linear transformation (Scatchard) of the data set in Panel A.

Fig. 6 depicts the results of a competitive binding assay run on the EPO agonist peptide (SEQ ID NO: 8) in competitive binding with [^{125}I]EPO to EBP beads (Panel A); and EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing either a human (Panel B) or murine EPO receptor (Panel C).

Fig. 7 is a graphic representation of the results of the exhypoxic mouse bioassay; stimulation f the incorporation of [^{59}Fe] into nascent red blood cells by EPO, peptide (SEQ ID NO: 8) (Panel A) and peptide (SEQ ID NO: 8) dimer (Panel B).

Fig. 8 demonstrates the effect of PEG dimerization of peptide (SEQ ID NO: 18) activity in EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing a human EPO receptor.

Fig. 9 provides the sequences of representative monomeric peptides of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to peptide dimers which behave as cell surface receptor agonists, dimers which exhibit binding and signal initiation of growth-factor-type receptors. Sometimes called cell-surface receptors, growth-factor-type receptors or dimerization-mediated activator-receptors, these are a class of molecules which are understood to be activated by ligand-induced or ligand stabilized dimerization. Agonists of such receptors typically include large polypeptide hormones including the cytokines, insulin and various other growth or differentiation factors. The agonists are understood to induce dimerization of the receptor and thereby effect signal initiation. Such agonists are believed to effectively cross-link two receptors resulting in the repositioning of cytoplasmic tails which may directly or indirectly effect phosphorylation of the cytoplasmic tails and activation of a signaling pathway.

The present invention specifically includes those molecules which behave as agonists of cell-surface receptors when dimerized in accordance with this invention. Such dimer agonists can include 'monomeric' units which exhibit agonist or antagonist activity for the related receptor molecule and may be the same or different. The dimers are preferably peptides but may alternatively be small molecule pharmacophores. These molecules when dimerized exhibit agonist activity of

cell-surface receptors in vitro and in vivo. Such receptors include, for example, EPO, GM-CSF, G-CSF, M-CSF, GH, EGF, PDGF, VEGF, Insulin and FGF. Other receptors which are activated by heterodimerization or multimerization may also be subject to activation by this mechanism including, IL-3, IL-5, IL-6, IL-2 and TPO. The dimers of the present invention have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 amino acid residues in length. In a preferred embodiment, these monomeric peptide units comprise a core sequence of amino acids $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

More preferably, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3), where each amino acid is indicated by standard one letter abbreviation; each X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

In a more preferred embodiment, both X_3 and X_8 will be C and thus, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 4). More preferably, the monomeric peptide unit comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 5), where X_4 can be R or H; X_5 can be F or M; X_6 can be I, L, T, M, or V; X_7 is D or V; X_9 can be G, K, L, Q, R, S, or T; and X_{10} can be A, G, P, R, or Y. In a most preferred embodiment, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 6), where X_1 can be D, E, L, N, S, T, or V; X_2 can be A, H, K, L, M, S, or T; X_4 is R or H; X_9 can be K, R, S, or T; and X_{10} is P. Particularly preferred monomeric peptide units of the present dimers include:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);

GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18).

The dimer peptides of the present invention exhibit increased biological potency in vitro and in vivo relative to the monomeric agonists from which the dimers are derived. Moreover, cell surface receptor antagonists can be 'converted' to cell surface receptor agonists in accordance with the present invention. Specifically, a cell surface receptor antagonist can be dimerized with PEG or another appropriate linker which permits mutual binding of the monomeric moieties with the receptors. As a result, the dimer exhibits effective binding to the target receptor and behaves as an agonist. Accordingly, the dimers of this invention demonstrate enhanced biological potency in vitro and in vivo relative to their monomeric forms.

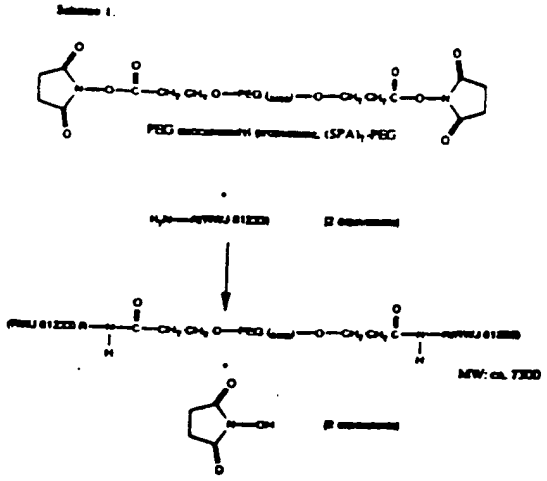
The dimer peptides of the present invention bind to and biologically activate the cell surface receptor or otherwise behave as agonists and are preferably formed by employing polyethylene glycol as a linker between the monomeric peptide units described herein. While other conventional chemical systems can

also be employed to form the dimer peptides of this invention including using other known polymeric compounds, pegylation is preferred.

The linking compounds of the present invention include any molecule which covalently binds the monomeric peptides at an appropriate distance or which otherwise effects dimerization of the particular cell surface receptor thereby initiating biological efficacy.

Starting with an appropriate synthetic peptide, containing a free amino group or other reactive site such as hydroxyls, carboxylic acids or sulfhydryls, the peptide is added in excess to a reaction mixture containing a corresponding reactive polymer. The polymer can be of a repeating nature such as polyethylene glycol, peptides, modified peptides or peptide analogs. Alternatively, the peptide can be dimerized on a small molecule scaffold such as activated benodiazepins, oxazolones, azalactones, aminimides or diketopiperazines. The most readily available linker of variable distance are ones based on linear unbranched polyethylene glycols.

The following is a schematic of a preferred preparatory methodology employing PEG succinimidyl propionate as the linker between the monomer units of the dimer peptides.



Dimerization and especially pegylation in a head-to-head (amino to amino terminus) or head-to-tail (amino to carboxyl terminus) configuration is preferred relative to internal covalent binding of the monomeric peptides. The 'monomer' units of the dimer peptides of the present invention can be the same or different, although the same are preferred.

The monomeric peptides which are used to form the dimers of the present invention can be prepared by classical chemical methods well known in the art. The standard methods include, for example, exclusive solid phase synthesis and recombinant DNA technology. See, e.g. Merrifield (1963) J. Am. Chem. Soc. 85:2149. Solid phase synthesis is typically commenced from the C-terminal end of the peptide using an α-amino protected resin. A suitable starting material can be prepared by attaching the required α-amino acid to a chloromethylated resin (such as BIO-BEADS SX-1, Bio Rad Laboratories, Richmond, CA), a hydroxymethyl resin, (described by Bodonszky et al. (1966) Chem. Ind.

(London) 38:1597) or a benzhydrylamine resin (described by Pietta and Marshall (1970) Chem. Commn. 650).

The α-amino protecting groups are those known to be useful in the art of stepwise synthesis or peptides. Included are acyl type protecting groups (e.g. formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups (e.g. benzyloxycarboyl (Cbz) and substituted Cbz), aliphatic urethane protecting groups (e.g., t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (e.g., benzyl and triphenylmethyl). The preferred X-amino protecting group is Fmoc. The side chain protecting group (typically ethers, esters, trityl, PMC, and the like) remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide.

The side chain protecting groups for Tyr include tetrahydropyranyl, tert-butyl, trityl, benzyl, Cbz, Z-Br-Cbz, and 2,5-dichlorobenzyl. The side chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl, and cyclohexyl. The sid chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl, and Cbz. The side chain protecting groups for Thr and Ser are benzyl. The side chain protecting groups for Arg include nitro, Tosy. (Tcs), Cbz, adamantyloxycarbonyl mesitoylsulfonyl (Mts), or

Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), Tos, or Boc.

After removal of the α-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. Each protected amino acid is generally reacted in about a 3-fold excess using an appropriate carboxyl group activator such as 2-(1H-benxotriazol-1-yl)-1,1,3,3tetramethyluronium hexafluorophosphate (HBTU) or dicyclohexylcarbodiimide (DCC) in solution of methylene chloride (CH_2CI_2), or dimethyl formamide (DMF) mixtures.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treating the mixture with a reagent such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF). These reagents not only cleave the peptide from the resin, but also cleave all remaining side chain protecting groups. When the chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the desired side chain protected amide or with an alkylamine to give a side chain protected alkylamid or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluorid to give the free amides, alkylamides, or dialkylamides.

These procedures can also be used to synthesize peptides in which amino acids other than the 20 naturally occurring, genetically encoded amino acids are substituted at one, two or more positions of any of the compounds of the invention. For instance, naphthylalanine can be substituted for tryptophan, facilitating synthesis. Other synthetic amino acids that can be substituted into the peptides of the present invention include L-hydroxypropyl, L-3, 4-dehydroxyphenylalanyl, δ amino acids such as L-δ-hydroxylysyl and D-δ-methylalanyl, L-α-methylalanyl, ß amino acids, and isoquinolyl. D-amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides of the present invention.

In another embodiment of the present invention, a method of enhancing the in vitro or in vivo biological potency of a cell surface receptor agonist is provided. This methodology is achieved by dimerizing the receptor agonist with a linker molecule, such as PEG, to form an appropriate spatial relationship between the monomeric peptide units of the dimer and thereby permitting each of the constituents of the dimers to bind to their receptors to achieve enhanced biological potency, i.e., to dimerize and thereby activate the receptors to induce the relevant biological activity of the particular cell-surface receptor, e.g. EPO-R. Biological activity can be measured by the skilled artisan in various in vitro and in vivo assays and as demonstrated in the examples of the present invention.

The peptide or molecule with binding affinity for a given receptor will have increased conformational

flexibility leading to fewer barriers to effective receptor interaction and subsequently receptor activation. This is also indicated for molecules which can bind but not activate a receptor subtype in that such molecules can become more effective inhibitors of ligand binding.

The present invention further provides a method for altering a cell-surface receptor antagonist, a molecule exhibiting receptor binding but no biological activity, to behave as a cell-surface receptor agonist in vitro or in vivo. This method is achieved by dimerizing the antagonist molecule with an appropriate linker molecule such as PEG, other polymerized molecules or a peptide. In a preferred embodiment, an EPO antagonist, i.e. a peptide exhibiting receptor binding but no biological EPO activity can be altered by dimerization to obtain a dimer which behaves as an EPO receptor agonist. Thus, for example, in the case of EPO-R these include the monomeric peptide units of the present dimers comprising a core sequence of general formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein, in (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When X_2 is present, i.e., when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to

delete the Y, tyrosine residue in SEQ ID NOS. 21 - 93. Such monomeric peptides can also be prepared by substitution of Y in the peptides of Fig. 9.

These molecules, demonstrate only binding activity in their 'monomeric' form, but exhibit agonist activity after dimerization with a linking compound such as PEG. Accordingly, the present method comprises identifying a monomeric peptide as herein defined which does not demonstrate biological activity and dimerizing that antagonist in accordance with the present invention to obtain a cell-surface-receptor agonist i.e., in dimeric form. Contacting the appropriate cell-surface receptor with the thus formed dimer activates, i.e. dimerizes such receptors and thus induces biological activity of the receptor. Such monomeric units as shown in Fig. 9 can be truncated from the N-terminus such as SCHFGPLTWVCK (SEQ ID NO: 18) to eliminate the tyrosine residue at position A of the formula $(AX_2)_mX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) or merely substituted with any of the remaining 19 naturally occurring amino acids or with other than a non-naturally occurring aromatic amino acid analog. In accordance with the present invention it has been determined that the tyrosine residue at position A of the foregoing formula is critical to biological activity of the monomer peptide. Deletion or substitution of the tyrosine eliminates biological activity. When dimerized however the entity exhibits enhanced biological activity.

For example, tyrosine (Y) substituted in th formula $YX_2X_3X_4X_5GPX_7X_8$ (SEQ ID NO: 2) by p-

iodohydroxyphenylalanine, p-fluorohydroxyphenylalanine, p-amino-hydroxyphenylalanine act as EPO-R monomer agonists but substitution with threonine or alanine for tyrosine at position Y causes the monomer peptide to act as an EPO-R antagonist. However, when dimerized in accordance with the present invention, such dimers behave as EPO-R agonists. The monomeric peptide units identified at Fig. 9, for example, behave as EPO-R antagonists in the absence of tyrosine at position Y of the formula above. When such antagonists are dimerzied, the dimer behaves as an EPO-R agonist.

In a further embodiment of the present invention, pharmaceutical compositions comprising at least one of the dimers of this invention can be employed to therapeutically treat disorders resulting from deficiencies of biological factors such as EPO, GH, GM-CSF, G-CSF, EGF, PDGF, VEGF, insulin, FGF and the like. These pharmaceutical compositions may contain buffers, salts and other excipients to stabilize the composition or assist in the delivery of the dimerized molecules.

In a preferred embodiment, the present invention provides a method for treating disorders associated with a deficiency of EPO. The method is accomplished by administering at least one of the dimers identified herein for a time and under conditions sufficient to alleviate the symptoms of the disorder, i.e. sufficient to effect dimerization or biological activation of EPO receptors. In the case of EPO such methodology is useful in the treatment of end-stage renal failure/dialysis; anemia, especially associated

with AIDS or chronic inflammatory diseases such as rheumatoid arthritis and chronic bowel inflammation; auto-immune disease; and for boosting the red blood cell count of patient when necessary, e.g. prior to surgery or as pretreatment to transfusion. The dimers of the present invention which behave as EPO agonists can be used to activate megakaryocytes.

Since EPO has been shown to have a mitogenic and chemotactic effect on vascular endothelial cells as well as an effect on central cholinergic neurons (see, e.g., Amagnostou et al. (1990) Proc. Natl. Acad. Sci. USA 87:597805982 and Konishi et al. (1993) Brain Res. 609:29-35), the compounds of this invention can also be used to treat a variety of vascular disorders, such as promoting wound healing, growth of collateral coronary blood vessels (such as those that may occur after myocardial infarction), trauma, and post vascular graft treatment, and a variety of neurological disorders, generally characterized by low absolute levels of acetyl choline or low relative levels of acetyl choline as compared to other neuroactive substances e.g., neurotransmitters.

Accordingly, the present invention includes pharmaceutical compositions comprising, as an active ingredient, at least one of the peptide dimers of the present invention in association with a pharmaceutical carrier or diluent. The dimers of this invention can be administered by oral, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), transdermal (either passively or using iontophoresis or electroporation) or transmucosal

(nasal, vaginal, rectal, or sublingual) routes of administration in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pill, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as it normal practice, additional substances other than inert diluents, e.g., lubricating, agents such as magnesium stearate. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering, agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring and perfuming agents.

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting,

emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

Compositions for rectal or vaginal administration are preferably suppositories which may contain, in addition to the active substance, excipients such as cocoa butter or a suppository wax. Compositions for nasal or sublingual administration are also prepared with standard excipients well known in the art.

The dosage of active ingredient in the compositions of this invention may be varied; however, it is necessary that the amount of the active ingredient shall be such that a suitable dosage form is obtained. The selected dosage depends upon the desired therapeutic effect, on the route of administration, and on the duration of the treatment desired. Generally dosage levels of between 0.001 to 10 mg/kg of body weight daily are administered to mammals.

As can be appreciated from the disclosure above, the present invention has a wide variety of applications. Accordingly, the following examples are offered by way of illustration, not by way of limitation.

EXAMPLE 1

SDS-PAGE gels (10-20% gradient SDS-PAG plates, 84 x 70 x 1.0 mm, Integrated Separation Systems, Natick, MA) were stained with Coomasie Brilliant Blue R-250 (BioRad). A commercial preparation of activated difunctional polyethylene glycol (PEG-succinimidyl-propionate, SPA2, MW ca. 3400) was purchased from Shearwater Polymers, Huntsville, AL as was the monofunctional reagent, methoxy-PEG-succinimidyl-proprionate, MW ca 5000. Peptide (SEQ ID NO: 8) and all other peptides were obtained from the Peptide Synthesis Facility RWJ-PRI, La Jolla, CA or Quality Controlled Biochemical, Hopkinton MA. These peptides were cyclized via oxidation of their intramolecular cysteines, amidated at the C-terminus and mass confirmed by FAB-MS. All were Ellman Reaction negative. Tris base was obtained from BioRad, Hercules, CA. (DPDPB) and trifluoracetic acid (HPLC grade) were obtained from Pierce Chemical Co., Rockford IL.

Mono-PEG conjugation of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8)

This example describes the preparation of mono-PEG conjugates of peptide (SEQ ID NO: 8), using the monofunctional amine reactive polymer analog m-SPA-PEG to be used as a control in experiments described herein. The reaction was carried out with polymer in excess (ca. 3 fold) by resuspending 142.5 mg (0.0286 mmol, MW ca. 5000) of polymer in 4 ml PBS at pH 7.5 and adding 20 mg peptide (SEQ ID NO: 8) (0.0095 mmol, MW 2092) dissolved

in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. The reaction was subsequently adjusted to a final concentration 50 mM Tris by the addition of 1 M tris-HCl at pH 7.5. The reaction mixture was incubated on ice for one hour. Analytical HPLC suggested that there were two main reaction products of essentially equivalent magnitude which were not baseline resolved. Preparative HPLC (using the flatter gradient system described in Example 8) and conservative cuts resulted in collection of two product peaks eluting at ca 44 and 47 minutes. After lyophilization, 24.8 mg, 16.5 mg of each species was recovered, respectively. Mass spectral analysis of these two species demonstrated centroid masses of 7092 (peak 1) and 12036 (peak 2) indicating the coupling of one or two PEG molecules, respectively, to the peptide (Table I).

Tris inactivated polymer. Tris inactivated polymer was formed by incubation of 5 mM SPA2 polymer dissolved in PBS (Gibco, Gaithersburg, MD) with 50 mM tris-HCl, pH 7.5 added and used without further purification.

TABLE 1

Recovery Yield of Peptide Conjugation Reaction and Apparent Molecular Mass of Product

I.D. No.	Sequence	Mass	Conjugation Reagent	Main Product Mass (centroid m/z)	Yield (% of theoretical)
8	GGTYSCHFGPLTWVCKPQGG	2092	SPA2-PEG (MW ca. 3400)	7834	69
			m-SPA-PEG (MW ca. 5000)	7092 (peak 1)	-
				12036 (peak 2)	-
13	GGTYSCHFGPLTWVCKPQ	1978	SPA2-PEG	7560	54
20	Ac-GGTYSCHFGPLTWVCKPQGG	2133	SPA2-PEG	7862	30
14	GGLYACHMGPMTWVCQPLRG	2177	SPA2-PEG	7872	37
18	SCHFGPLTWVCK	1375	SPA2-PEG	6326	45

EXAMPLE 2

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8) (lot #1)

Examples 2 - 7 describe the dimerization of various peptides described by the present invention. The modification of peptide (SEQ ID NO: 8) was carried out by resuspending 25 mg (0.0071 mmol) of polymer in 4 ml PBS at pH 7.5, and adding a 3 fold molar excess of peptide (SEQ ID NO: 8) (0.0213 mmol, 44.5 mg, MW 2092) dissolved in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice. After 3 hours of incubation, an additional 7.5 mg (0.0036 mmol) of lyophilized peptide was added, resulting in a final ratio of 3.5 moles of peptide per each mole of SPA2. The mixture was incubated an additional 17 hours on ice. The reaction mixture was adjusted to a final concentration of 50 mM Tris by the addition of 1 M tris-HCl of pH 7.5 and incubated on ice for 1 hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. After preparative HPLC and lyophilization, 38 mg of PEG dimer was recovered. The theoretical yield for this experiment was 55 mg based on a calculated mass of 7600 mg/mmol for a yield of 69% (Table I).

EXAMPLE 3

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8) (lot #2)

The modification of peptide (SEQ ID NO: 8) was carried out by resuspending 25 mg (0.0071 mmol) of polymer in 4 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 8) (0.0213 mmol, 45.8 mg, MW 2092) dissolved in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 22 hours. At that time, the reaction was adjusted to a final concentration of 50 mM Tris by the addition of 1 M tris-HCl, pH 7.5. The reaction mixture was incubated on ice for 1 hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. After preparative HPLC and lyophilization, 37 mg of PEG dimer was recovered. The theoretical yield for this experiment was 55 mg based on a calculated mass of 7600 mg/mmol for a yield of 68% (Table I).

EXAMPLE 4

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13)

The modification of peptide (SEQ ID NO: 13) was carried out by resuspending 11.2 mg (0.0033 mmol) of polymer in 2.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 13) (0.010 mmol, 20 mg, MW 1978) dissolved in 0.25 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. At that time, 0.25 ml of 1 M tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for one hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. The main preparative reaction product peak eluted at ca 43 minutes. After preparative HPLC and lyophilization, 13.3 mg of PEG dimer was recovered. The theoretical yield for this experiment was 24.42 mg based on a calculated mass of 7400 mg/mmol for a yield of 54% (Table I).

EXAMPLE 5

PEG dimerization of peptide Ac-GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 20)

The modification of peptide (SEQ ID NO: 20) was carried out by resuspending 10.5 mg (0.0031 mmol) of polymer in 2.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 20) (0.0094 mmol, 20 mg, MW 2133) dissolved in 0.25 ml of 0.1% trifluoroacetic acid and the mixture incubated at 4°C for 28 hours. At that time, the reaction as monitored by HPLC was estimated to be approximately 30% complete, the temperature was shifted to ambient and an additional 27 hour incubation provided no net increase in product. Because of possible hydrolysis of the reactive polymer, an additional 5 mg of polymer was added and the incubation was continued for an additional 16 hours. At that time, 0.25 ml of 1 M tris-HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for an additional 1 hour. The sample was subjected to analytical and preparative HPLC using a flatter gradient system as described in Example 8. The main preparative reaction product peak eluting at ca 48 minutes. After preparative HPLC and lyophilization, 10.4 mg of PEG dimer was recovered. The theoretical yield for this experiment was 34.4 mg based on a calculated mass of 7650 mg/mmol for a yield of 30% (Table I).

EXAMPLE 6

PEG dimerization of peptide (SEQ ID NO: 14)

The modification of peptide (SEQ ID NO: 14) was carried out by resuspending 2.6 mg (0.00076 mmol) of polymer in 3.0 ml PBS at pH 7.5 and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 14) (0.00229 mmol, 5 mg, MW 2177) dissolved in 0.1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 26 hours. At that time, 0.25 ml of 1 M-tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for 1 hour. The sample was subjected to analytical and preparative HPLC using the flatter gradient system described in Example 8. The main preparative reaction product peak eluted at ca 46 minutes. After preparative HPLC and lyophilization, 2.2 mg of PEG dimer was recovered. The theoretical yield for this experiment was 24.42 mg based on a calculated mass of 7400 mg/mmol for a yield of 37% (Table I).

EXAMPLE 7

PEG dimerization of peptide (SEQ ID NO: 18)

The modification of peptide (SEQ ID NO: 18) was carried out by resuspending 1.2 mg (0.00036 mmol) of polymer in 0.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of the peptide (0.0011 mmol, 1.5 mg, MW 2177) dissolved in 0.05 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. At that time, 0.1 ml of 1 M tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for 1 hour. The sample was subjected to purification using an analytical HPLC system as described in Example 8. The main reaction product peak eluted at ca 38 minutes. After preparative HPLC and lyophilization, 1 mg of PEG dimer was recovered. The theoretical yield for this experiment was 2.2 mg based on a calculated mass of 6150 mg/mmol for a yield of 45% (Table I).

EXAMPLE 8

Analytical and Preparative HPLC analysis

The accumulation of the dimers described above in Examples 1-7 was monitored by analytical reverse phase HPLC. The analysis was carried out using a Vydac C-18 Protein-Peptide column (0.46 x 25 cm, part no. 218TP54) and a Rainin Gradient HPLC system fitted with a Dynamax dual wavelength detector. At injection, the column was equilibrated in 0.1% TFA in dH_2O and was developed with a 45 minute linear gradient (0-100%) of acetonitrile (ACN) containing 0.1% TFA beginning at 10 minutes after injection. The flow rate was held constant at 1 ml/min. Under these analytical conditions, the SPA2 polymer and tris inactivated polymer did not appear to bind the column while a major reaction product with a retention time (37 minutes) was identified (Figure 1). Peptide (SEQ ID NO: 8) demonstrated a retention time of 35 minutes and the excess peptide utilized in the reaction was clearly distinguished from the nascent reaction products.

The main product reaction product peak was purified by preparative reverse phase HPLC on the same chromatographic system using a Vydac C-18 Protein-Peptide column (2.2 x 25 cm, part no. 218TP15022). Injection of the reaction mix (6 ml) occurred with the column equilibrated at 80:20, H_2O:ACN (both containing 0.1% TFA) at a constant flow rate of 8 ml/min. After a 20 minute wash, the column was developed by application of a linear gradient of 100% ACN/0/1% TFA over 60

minutes. The major product peak eluting at 48 minutes was collected and lyophilized (Figure 2). These elution conditions were subsequently modified to improve the resolution of some conjugation products peptide (SEQ ID NO: 20), mPEG-peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 14) from reaction by products. This was accomplished by application of a flatter linear gradient of 20-80% B over 60 minutes. The variation in retention time due to different peptides and elution condition is described as part of each synthesis example. The materials recovered from the main product peak from each reaction were subsequently analyzed by analytical reverse phase HPLC, MALDI-TOF mass spectrometry, EPO competitive binding potential and for in vitro bioactivity.

The activated PEG used in these experiments has an approximate molecular weight of 3400 and has amine reactive succinimidyl groups on either end of the difunctional linear polymer. This reactivity was employed to couple two equivalents of peptide (SEQ ID NO: 8) (MW= 2092) to the polymer with the concomitant liberation of two succinimidyl moieties resulting in a dimeric product as shown in Scheme I. Peptide (SEQ ID NO: 8) contains two potentially reactive amines, one at the N-terminus of the peptide and one in the side chain of the single lysine within the peptide sequence, so that a number of different connectivities between the two molecules was possible.

MALDI-TOF mass spectral analysis was supportive of the presence of the expected dimeric product (Figure 3) as indicated by a predominant species.

with a centroid mass of 7661. This data shows that the dimeric product described in the present invention was produced using the methods described herein.

EXAMPLE 9

EBP (EPO Binding Protein) Dimerization

This example demonstrates the interaction of peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 16), peptide (SEQ ID NO: 18) and peptide (SEQ ID NO: 13) with EPO binding protein (EBP) using a bifunctional sulphydryl reactive crosslinker, (1,4-Di-[3'-(2'-pyridyldithio)propionamido]butane DPDPB.

To explore the interaction of peptide (SEQ ID NO: 8) with EBP, a bifunctional sulphydryl reactive crosslinker (DPDPB) was used in an attempt to stabilize a mimetic dependent dimeric structure. Control experiments demonstrated that the crosslinker does not inactivate the EPO binding potential of EBP or the proliferative properties of peptide (SEQ ID NO: 8). As shown in Figure 4, a dimeric EBP product was formed by co-incubation of the peptide, peptide (SEQ ID NO: 8), DPDPB and EBP. This data shows the ability of the peptide (SEQ ID NO: 8) to mediate formation of a solubl receptor dimer. To further explore this question, peptides (SEQ ID NO: 13), (SEQ ID NO: 16) and (SEQ ID NO: 18) were examined for their ability to mediate dimerization. As shown in Figure 4, lanes 7A and 8A, when peptide (SEQ ID NO: 13) was truncated at the carboxyl terminus, it retained good in vitro bioactivity and improved in vivo bioactivity, resulting in a crosslinking signal similar to peptide (SEQ ID NO: 8). However, peptide (SEQ ID NO: 18) did not appear to stabilize the dimerization signal (Figure 4, lanes 9A

and 10A) whereas peptide (SEQ ID NO: 16) (Figure 4, lanes 5A and 6A) gave a strong dimerization band. These two peptides differ by a single N-terminal tyrosine residue and display a similar profile in the in vitro proliferation assay with peptide (SEQ ID NO: 18) being inactive. Peptide (SEQ ID NO: 16) has an ED_{50} of 3 μM on murine receptor cells. Both peptides have similar IC_{50} values indicating that they both retain binding activity. These results demonstrate that EBP dimerization is a property of the EPO peptide series and that the presence of the tyrosine is critical for this activity and that this corresponds to in vitro bioactivity.

EXAMPLE 10

IMMOBILIZED EBP BASED [^{125}I]EPO COMPETITION BINDING ASSAY

This study examined the binding capacity of the EPO PEG dimers to bind EPO receptors.

The extracellular domain of the human erythropoietin receptor (EPO binding protein, EBP) was expressed and overproduced in E. coli. As with many other recombinant eukaryotic proteins produced in E. coli, the protein appeared as an insoluble product in laboratory scale fermentations and was refolded and purified to obtain active protein. EPO binding protein produced by this method contains one free sulfhydryl group which can be modified without effecting the solution phase binding of ligand. In order to immobilize the EPO binding protein for equilibrium binding analysis and for competition binding assay, the EPO binding protein was covalently attached to agarose beads.

The iodoacetyl activation chemistry of Sufolink beads (Pierce Chemical Co, Rockford, IL) is specific for free thiols and assures that the linkage is not easily reversible. EBP-Sulfolink beads were made as follows: SulfoLink gel suspension (10 ml) was mixed with of coupling buffer (40 ml: 50 mM Tris, pH 8.3, 5 mM EDTA) and the gel was allowed to settle. The supernatant was removed and the EPO binding protein (0.3-1 mg/ml in coupling buffer) to be bound was add d directly to the washed beads. The mixture was rocked

gently for 30 minutes at room temperature and the beads were allowed to settle for 1 hour at room temperature. The supernatant was removed and retained. The beads were washed twice with 20 ml of coupling buffer. The washes were recovered as well. The beads were then treated with 20 ml of 0.05 M cysteine for 30 minutes at room temperature to block unbound sites. Finally, the beads were washed with 50 ml of 1 M NaCl, then with 30 ml of PBS, and resuspended in 20 ml of PBS and stored at 4°C. The amount of EBP which was covalently bound to the beads was determined by comparing the OD_{280} of the original EBP solution to the total OD_{280} recovered in the reaction supernatant and the two 20 ml washes. Typically, 40-60% of the applied EBP remains associated with the beads.

Binding assays were initiated by the addition of EPO binding protein beads (50 µl) to individual reaction tubes. Total binding was measured in tubes containing 0.3-30 nM [^{125}I]EPO (NEN Research Products, Boston MA, 100 µCi/µg). For determination of non-specific binding, unlabelled EPO was added at a level of 1000 fold in excess of the corresponding [^{125}I]EPO concentration. Each reaction volume was brought to 500 µl with binding buffer (PBS/0.2% BSA). The tubes were incubated for five hours (a time period experimentally determined as adequate for the establishment of equilibrium) at room temperature with gentle rocking. After five hours, each reaction mixtur was passed through a 1 ml pipet tip plugged with glass wool. The tubes were washed with 1 ml wash buffer (PBS/ 5% BSA) and this volume as well as 2 additional 1 ml

washes were passed through the pipet tip and collected for determination of the free EPO concentration. Equilibrium binding analysis of the specific association of [^{125}I]EPO with EPO mimetic binding proteins immobilized on these agarose beads indicates a Kd of 5 nM ± 2 based on a linear transformation (Scatchard) of the binding isotherm (Figure 5).

Competitive binding analysis assays of candidate peptides and dimer peptides were performed as outlined below. Individual peptides were dissolved in DMSO to prepare a stock solution 1 mM. Dimer peptides were contained within PBS at a concentration of 5 mM. All reaction tubes (in duplicate) contained 50 μL of EBP beads, 0.5 nM [^{125}I]EPO and 0-500 μM peptide in a total of 500 μL binding buffer.

The final concentration of DMSO was adjusted to 2.5% in all peptide assay tubes. At this concentration DMSO has no detectable effect since an examination of the sensitivity of the assay to DMSO demonstrated that concentrations of up to 25% DMSO (V/V) had no deleterious effect on binding. Non-specific binding was measured in each individual assay by inclusion of tubes containing a large excess of unlabelled EPO (1000 nM). Initial assay points with no added peptide were included in each assay to determine total binding. Binding mixtures were incubated overnight at room temperature with gentle rocking. The beads were then collected using Micro-columns (Isolab, Inc.) and washed with 3 mL of wash buffer. The columns containing the washed beads were placed in 12 x 75 mm glass tubes and bound radioactivity levels determined in

a gamma counter. The amount of bound [^{125}I]EPO was expressed as a percentage of the control (total=100%) binding and plotted versus the peptide concentration after correction for non-specific binding. The IC_{50} was defined as the concentration of the analyte which reduced the binding of [^{125}I]EPO to the EBP beads by 50%. All data are reported as relative to peptide (SEQ ID NO: 8) which demonstrated an IC_{50} of 5 μM.

Competitive binding analysis revealed an IC_{50} of 20 μM for the purified dimer, a value four fold greater than peptide (SEQ ID NO: 8) in the same assay (Figure 5 and Table II). Polymer alone, which was inactivated by treatment with Tris-HCl, demonstrated a detectable competition binding signal but this signal was modest (<10%) at the IC_{50} of the PEG-peptide (SEQ ID NO: 8) dimer.

TABLE II

Table II. Binding and Cell Proliferation Studies

Id	Relative Binding[1]	EPO-ED_{50} (μM)[2]	
		murine receptor	truncated human receptor
D. No. 8	1	0.1	0.09
ris inact.polymer	60	IA[3]	IA
q. I.D. No. 8 covalent dimer #1	4	0.01 (10X)	0.0015 (60X)
q. I.D. No. 8 covalent dimer #2	3	0.01 (10X)	0.002 (45X)
D. No. 13	1.6	0.08	0.02
q. I.D. No. 13 covalent dimer	3	0.01 (8X)	0.002 (10X)
D. No. 20 (N-acetyl)	4	0.03	0.06
q. I.D. No. 20 covalent dimer	12	0.2 (-7X)	0.05
D. No. 14 (terminal NH_2)	0.6	0.1	0.08
q. I.D. No. 14 covalent dimer	-	0.006 (16X)	0.001 (80X)

[1] Amount required to achieve one half maximal level of EPO dependent proliferation (11pM)
[2] Binding relative to Seq. I.D. No. 8
[3] Inactive
[4] Note that all peptides are cyclic and were analyzed as COOH terminal amides (-$CONH_2$)

EXAMPLE 11

EPO DEPENDENT CELL PROLIFERATION ASSAYS

This example shows the improved potency of PEG-EPO peptide dimers to EPO receptors in human and murine cell lines.

Cell line FDC-P1/ER, an EPO-dependent line expressing the murine EPO receptor, was grown and maintained as described previously (Carroll et al. 1991). Also employed was cell line FDC-P1/trER expressing a functional truncated human EPO receptor (missing the C-terminal 40 amino acids). Both cell lines exhibit EPO dependent cellular proliferation. Briefly, cells were maintained in RPMI 1640 media (Gibco/BRL) containing 10% heat-inactivated fetal calf serum and 10 units/ml of recombinant human EPO. For the cellular proliferation assay, FDC-P1/ER or FDC-P1/trER cells were grown to stationary phase, centrifuged, washed with RPMI 1640 media (no EPO), and plated in EPO minus media for 24 hr.

After 24 hours, the cells were counted, resuspended at 800,000 cells/ml and dispensed at 40,000 cells/well. Stock solutions of the peptide dimer (5 mM in PBS) and peptide (10 mM in DMSO) were prepared and dispensed in triplicate to final concentrations of 1 x 10^{-10} M through 1 x 10^{-5} M and adjusted to a final volume of 0.2 ml. Final DMSO concentrations of 0.1% (V/V, maximal) or less were found to have no cellular toxicity or stimulatory effects. A standard EPO dose response curve was generated with each assay series.

After a 42 hr incubation at 37°C (ca. 2 cell doublings) 1μCi/well of [^{3}H] thymidine was added and the incubation continued for 6 hr at which time the cells were harvested and counted to assess [^{3}H]thymidine incorporation as a measure of cell proliferation. Results are expressed as the amount of peptide or dimer peptide necessary to yield one half of the maximal activity obtained with recombinant EPO.

As shown in Figure 5 and Table II, the initial lot of PEG-peptide (SEQ ID NO: 8) dimer demonstrated ED_{50} values of 0.01 μm and 0.0015 μm in EPO responsive cell lines containing the murine or human EPO receptor, respectively. In both cell lines, the parent peptide, peptide (SEQ ID NO: 8), demonstrated an ED_{50} of 0.1 μM, indicating an increase in potency of 10 fold in the murine receptor line and almost 60 fold in the human receptor containing cells. Thus, the dimer was clearly more potent in murine and human lines than the peptides themselves. This was confirmed by generation of a second synthesis lot of PEG-peptide (SEQ ID NO: 8) dimer which resulted in a 10 and 45 fold increase in potency in the murine and human lines, respectively. Polymer alone, which was inactivated by treatment with Tris-HCl, demonstrated no activity in the cell proliferation assay.

A second EPO mimetic peptide, peptide (SEQ ID NO: 13), with the sequence GGTYSCHFGPLTWVCKPQ, was also subjected to a similar PEG dimerization protocol as that described above for peptide (SEQ ID NO: 8). The dimer product of PEG-peptide (SEQ ID NO: 13) is also more active than the unconjugated parent compound (Table II).

Both of these dimer peptides have ultimate ED_{50} values near 0.002μM. In spite of this more modest increase, the experimental evidence clearly indicates that the dimerization of these peptides with PEG results in improved potency.

EXAMPLE 12

To further examine the connectivity of the peptides of the present invention to PEG, peptide molecules, which contained only an internal lysine group were used peptide (SEQ ID NO: 8) analog acetylated at the N-terminus peptide (SEQ ID NO: 20) and a sequence analog peptide (SEQ ID NO: 14) which only had a reactive N-terminal amine were PEG dimerized. In vitro proliferation data of these compounds suggest that potential dimerization through the free amino terminus has the most profound effect on bioactivity giving rise to a species about 80 fold more active than the monomeric parent peptide (SEQ ID NO: 14) dimer. Conjugation through the lysine side chain had no real effect on activity peptide (SEQ ID NO: 20) as did mono-PEG or di-PEG conjugation (Table III). This data indicates that the creation of a head to head dimer (both peptides attached through the N-terminus) using a PEG linker greatly enhances the potency of EPO peptides and approaches a level almost two logs greater than the free parent peptide. Further, this effect was not observed upon simple covalent attachment of linear PEG to peptide (SEQ ID NO: 8) indicating that dimerization is a critical determinant for this increased activity.

TABLE III

Table III. Binding and Cell Proliferation Studies mPEG/

Compound	Relative Binding[3]	EPO-ED_{50} (μM)[*]	
		murine receptor	truncated human receptor
Seq. I.D. No. 8	1	0.1	0.09
mPEG/Seq. I.D. No. 8, peak #1	60	2	0.1
mPEG/Seq. I.D. No. 8, peak #2	>40	1	0.4

[*] Amount required to achieve the half maximal level of EPO dependent proliferation (11pM)
[1]ND=Not determined
[2]A=inactive
[3]Binding relative to Seq. I.D. No. 8

Note that all peptides are cyclic and were analyzed as COOH terminal amides (-$CONH_2$)

EXAMPLE 13

Polycythemic Exhyposic Mouse Bioassay.

This study demonstrates the ability of peptide (SEQ ID NO: 8)/PEG-dimers to retain in vivo bioactivity. Peptides were assayed for in vivo activity in the polycythemic mouse bioassay adapted from the method described by Cotes and Bangham (1961), Nature 191: 1065-1067. BDF1 mice were allowed to acclimate to ambient conditions for 7-10 days. Body weights were determined for all animals. Low weight animals (<15 grams) were not used. Mice were introduced to hypobaric chambers with a 24 hour conditioning cycle consisting of 0.40% +/- 0.02 atm. for 18 hours followed by 6 hours at ambient pressure for a total of 14 days. Following the 14 day period, mice were placed in ambient pressure for 72 hours prior to dosing. Test samples or recombinant Human Erythropoietin (rHuEPO) standards were diluted in an assay vehicle consisting of Phosphate Buffered Saline (PBS)-0.1% Bovine Serum Albumin (BSA). Peptide sample stock solutions (excluding peptide dimers) were first solubilized in dimethyl sulfoxide (DMSO). Control groups included one group of vehicle alone, and one group of (DMSO) at final concentration of 1%.

Each dose group contained 10 mice. Mice were injected subcutaneously (scruff of neck) with 0.5 ml of the appropriate sample. Forty eight hours following the sample injection, the mice were administered an intraperitoneal injection of 0.2 ml of [^{59}Fe]

(approximately 18.0 milliCuries/milligram, Dupont, NEN) and 0.75 microCuries/Mouse.

Mouse body weights were determined twenty four hours following [^{59}Fe] administration and the mice were sacrificed forty eight hours following the [^{59}Fe] injection. Blood was collected from each animal by cardiac puncture and hematocrits were determined (heparin was used as the anticoagulant). Each blood sample (0.2 ml) was analyzed for [^{59}Fe] incorporation using a Packard gamma counter. Non-responder mice (i.e.,those mice with radioactive incorporation less than the negative control group) were eliminated from the appropriate data set. Mice that had hematocrit values less than 53% were also eliminated.

This assay examined the ability of an exogenously administered compound to induce new red blood cell synthesis, or in other words to function as EPO or an EPO mimetic. The results are derived from sets of 10 animals for each experimental dose. As shown in Figure 7 and Table IV, the data suggests that on a mole equivalent basis, peptide (SEQ ID NO: 8)/PEG-dimer is about 10 fold more active than peptide (SEQ ID NO: 8) monomer. These results are consistent with <u>in vitro</u> results in which increased potency values of 10 fold was observed on murine EPO-R bearing cells.

TABLE IV

Table 4. Exhpoxic Mouse Bioassay Study of PEG Dimer Acivity

Compound	Amount required for equivalency to 0.025U EPO (nmol) n=10
Seq. I.D. No. 8	3.8

EXAMPLE 14

This example shows that an inactive truncation analog of peptide (SEQ ID NO: 8), which lacks the critical tyrosine peptide (SEQ ID NO: 18), (SCHFGPLTWVCK), can be converted to an agonist on the human EPO receptor cell line by PEG dimerization. In this experiment, a 10^{-5} M concentration of the parent peptide had no activity above background while the dimeric peptide exhibited a level of proliferation twice as many cpm as background. As shown in Figure 8, the peptide alone (open squares) did not induce proliferation of the EPO responsive cells but upon PEG dimerization (open diamonds) a significant agonist effect was observed. Approximately twice as many cpm incorporated over non-stimulated cells at 10^{-5} M added peptide dimer. The replicate error bars represent the standard deviation of three assay points per concentration of peptide or peptide dimer.

Exhypoxic polycythemic mouse bioassay:

PEG dimer and the monomer parent peptide RWJ 61718 were compared in the exhypoxic mouse bioassay (Table 1). This peptide exhibited an 80 fold increase in in vitro activity upon dimerization. Murine studies (in vivo) of the activity of the dimer compared to the monomer peptide revealed a 250 fold increase in activity of RWJ 61718 upon dimerization. Cellular proliferation studies on this dimer peptide in murine receptor containing cells demonstrated a 16 fold increase over the monomer indicating that the 250 fold increase in vivo might be attributable to other factors such as altered metabolism or prolonged circulatory half-life which occur upon PEG dimerization of the peptide sequence. Thus, in addition to the effect of dimerization alone, the PEG modification has an effect which impacts in vivo activity and may be specific to individual peptide sequences.

Cell associated EPO receptor competition binding assay.

A competitive binding analysis of the ability of selected monomer peptides and their cognate dimer products to compete with radiolabelled EPO binding for cell associated human EPO receptors was performed Erythropoietin Receptor Competition Binding Analysis was preformed as follows. TF-1 cells were maintained in RPMI 1640, 10% fetal calf serum, 1% L-glutamine, 1% penicillin, 0.1% streptomycin and 1 ng/ml of GM-CSF.[125]-EPO was obtained from NEN Research Products. Cells were centrifuged and washed 1 x with binding buffer (RPMI 1640, 5% BSA, 25 mM Hepes, pH 7.5, 0.02% sodium azide) resuspended in binding buffer, and counted using trypan lue as an indicator of viability. Each reaction contained approximately 5 x 10^5 cells, [125]-EPO (0.5 nM), no competitor or peptide or dimer preparation in a final volume of 200 µl. The binding reactions (in duplicate) were incubated overnight at 4°C. Following binding, the tubes were centrifuged at 12,000 rpm for 1 min at 4°C in a

refrigerated centrifuge. The supernatant was removed, the cell pellet resuspended in 100 µl of binding buffer, and the cell suspension layered onto 0.7 ml of bovine calf serum. The tubes were centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was removed, the bottom of the tubes snipped off, and the cell pellets counted in a Micromedic ME plus gamma counter. Non-specific binding was determined by incubating cells with [125]-EPO and a 100-fold excess of non-radioactive EPO. These data demonstrate increases in apparent binding competitive affinity of 3.0 fold, 3.2 fold and 80 fold for peptides RWJ 61233, RWJ 61596 and RWJ 61718, respectively (Table 2). In vivo proliferation studies with these peptides and their dimer derivatives reveal increases in potency of ea. 50 fold, 10 fold and 80 fold, respectively, indicating that the magnitude of increased binding affinity is exceeded by the functional potency of the peptide for two of the three species. Thus, the effect of dimerization and subsequent increase in activity may be one in which the efficiency of receptor stimulation is improved by limiting the lateral diffusion of the receptors away from a binding event. Peptide dimerization therefore likely results in entropic rather than enthalpic gains upon mimetic ligand-receptor association for some peptide dimer sequences.

Unlike the EBP-bead EPO competitive binding assay where peptide dimerization negatively impacted the ability of PEG dimer peptides to compete for receptor binding, the ability to compete for cell associated receptors is increased by dimerization. This may be due to the ability of the cell associated receptor to dimerize while the immobilized EBP monomer likely cannot.

Conversion of inactive to active peptide RWJ 61177 was further studied. An improved and expanded study was performed which confirmed our earlier observation of conversion to an active peptide (Figure 6, Panel D).

TABLE IV. EXHYPOXIC MOUSE BIOASSAY STUDY OF PEG DIMER ACTIVITY

Compound	Amount required for equivalency to 0.025 U EPO (nmol) n=10
RWJ 61233 (seq. ID #8)	3.8
SAP2/61233, covalent dimer	0.28
RWJ 61718 (seq. ID #14)	18
SAP2/61718, covalent dimer	0.07

TABLE V. EPO COMPETITIVE BINDING ANALYSIS OF CELL ASSOCIATED RECEPTORS

Compound	IC_{50} (μM)
RWJ 61233 (seq ID #8)	3.8
SAP2/61233, covalent dimer	0.28
RWJ 61718 (seq ID#14)	18
SAP2/61718, covalent dimer	0.07

SEQUENCE LISTING

NERAL INFORMATION:

i) APPLICANT: Johnson, Dana L
Zivin, Robert A

.i) TITLE OF INVENTION: AGONIST PEPTIDE DIMERS

.i) NUMBER OF SEQUENCES: 93

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/484,135
(B) FILING DATE: 07-JUN-1995
(C) CLASSIFICATION:

.ii) ATTORNEY/AGENT INFORMATION:
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INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

c) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos2) can be R,H,L or W; Xaa(Pos3) can be M,F or I; Xaa(Pos6) can be any one of the 20 L-amino acids or the stereoisomeric D-amino acids; Xaa(Pos9) can be D,E,I,L or V; and Xaa(Pos10) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos1) or Xaa(Pos10) is C or Hoc"

i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

aa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
5 10

NFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Xaa(Pos2) and Xaa(Pos8) can be any on of the 20 L-amino acids; Xaa(Pos3) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos4) can be R,H,L or W; Xaa(Pos5) can be M,F or I; Xaa(Pos11) can be D,E,I,L or V; and Xaa(Pos12) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos3) or Xaa(Pos12) is C or Hoc"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
1 5 10

INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

:) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1), Xaa(Pos 3), Xaa(Pos9), Xaa(Pos14), Xaa(Pos15) and Xaa(Pos16) can be any one of 20 L-amino acids; Xaa(Pos4) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos5) can be R,H,L or W; Xaa(Pos6) can be M,F or I; Xaa(Pos12) can be D,E,I,L or V; and Xaa(Pos13) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos4) or Xaa(Pos13) is C or Hoc"

i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

aa Tyr Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa Xaa Xaa Xaa
5 10 15

;FORMATION FOR SEQ ID NO:4:

.i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Xaa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
1 5 10 15

NFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1), Xaa(Pos3) and Xaa(Pos16) can be any one of the 20 L-amino acids; Xaa(Pos5) can be R or H; Xaa(Pos6) can be F or M; Xaa(Pos9) can be I,L,T,M or V; Xaa(Pos12) can be D or V; Xaa(Pos14) can be G,T,L,Q,R,S or T; Xaa(Pos15) can be A,G,P,R or Y"

i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

aa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
5 10 15

FORMATION FOR SEQ ID NO:6:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

.x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be D,E,L,N,S,T or V; Xaa(Pos3) can be A,H,K,L,M,S or T; Xaa(Pos5) can be R or H; Xaa(Pos6) can be F or M; Xaa(Pos9) can be I,L,T,M or V; Xaa(Pos12) can be D or V; Xaa(Pos14) can be K,R,S or T; Xaa(Pos15) is P and Xaa(Pos16) can be any one of the 20 L-amino acids"

xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Xaa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
1 5 10 15

NFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

'ORMATION FOR SEQ ID NO:8:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ly Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

ro Gln Gly Gly
20

IFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Gly Asp Tyr His Cys Arg Met Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Leu Gly Gly
20

:NFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Gly Asn Tyr Met Ala His Met Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly

INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

:) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ly Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

ro Gln

FORMATION FOR SEQ ID NO:14:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Gly Leu Tyr Ala Cys His Met Gly Pro Met Thr Trp Val Cys Gln
1 5 10 15

Pro Leu Arg Gly
20

NFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Ile Ala Gln Tyr Ile Cys Tyr Met Gly Pro Glu Thr Trp Glu Cys
1 5 10 15

Arg Pro Ser Pro Lys Ala
20

FORMATION FOR SEQ ID NO:16:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

hr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10

FORMATION FOR SEQ ID NO:17:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Tyr Cys His Phe Gly Pro Leu Thr Trp Val Cys
1 5 10

NFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10

INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.) MOLECULE TYPE: peptide

c) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be any one of the 20 L-amino acids; except that Xaa(Pos1) may or may not be Y and Xaa(Pos1) may be any non-naturally occurring aromatic acid analog when Xaa(Pos1) is Y. Xaa(Pos2) and Xaa(Pos8) can be any one of the 20 L-amino acids; Xaa(Pos3) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos4) can be R,H,L or W; Xaa(Pos5) can be M,F or I; Xaa(Pos11) can be D,E,I,L or V and Xaa(Pos12) can be C,A,α-amino-γ-bromobutyric acid or Hoc provided that either Xaa(Pos3) or Xaa(Pos12) is C or Hoc"

:i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Xaa Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
1 5 10

NFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Gln Gly Gly
20

INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ly Gly Thr Tyr Arg Cys Ser Met Gly Pro Met Thr Trp Val Cys Leu
5 10 15

ro Met Gly Gly
20

:FORMATION FOR SEQ ID NO:22:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Gly Met Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Gly
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Gly Trp Ala Trp Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Ala His Gly Gly
20

FORMATION FOR SEQ ID NO:24:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

ti) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gly Gly Met Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Ile
1 5 10 15

Pro Tyr Gly Gly
20

NFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gly Gly Glu Tyr Lys Cys Tyr Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Pro Glu Gly Gly
20

INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
5 10 15

.la Thr Gly Gly
20

IFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gly Gly Asn Tyr Leu Cys Arg Phe Gly Pro Gly Thr Trp Asp Cys Thr
1 5 10 15

Gly Phe Arg Gly
20

:NFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Gly Asn Tyr Val Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ly Gly Lys Asp Val Cys Arg Met Gly Pro Ile Thr Trp Asp Cys Arg
5 10 15

er Thr Gly Gly
20

IFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Gly Gly Ser Tyr Leu Cys Arg Met Gly Pro Thr Thr Trp Leu Cys Thr
1 5 10 15

Ala Gln Arg Gly Gly Gly Asn
20

INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Gly Gly Asn Tyr Leu Cys Arg Met Gly Pro Ala Thr Trp Val Cys Gly
1 5 10 15

Arg Met Gly Gly
20

FORMATION FOR SEQ ID NO:32:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

;ly Gly Glu Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Val Cys Gln
1 5 10 15

Tyr Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
1 5 10 15

Ala Thr Arg Gly
20

INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Gly Gly Val Tyr Val Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Thr
1 5 10 15

Ala Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Gly Gly Glu Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Ser
1 5 10 15

Pro Thr Gly Gly
20

INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Gly Gly Glu Tyr Leu Cys Arg Met Gly Pro Ile Thr Trp Val Cys Glu
1 5 10 15

Arg Tyr Gly Gly
20

INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ly Gly Asn Tyr Ile Cys Arg Met Gly Pro Met Thr Trp Val Cys Thr
5 10 15

la His Gly Gly
20

FORMATION FOR SEQ ID NO:38:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gly Gly Asp Tyr Leu Cys Arg Met Gly Pro Ala Thr Trp Val Cys Gly
1 5 10 15

Arg Met Gly Gly
20

NFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

FORMATION FOR SEQ ID NO:40:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ly Gly Leu Tyr Ser Cys Arg Met Gly Pro Ile Thr Trp Val Cys Thr
5 10 15

ys Ala Gly Gly
20

FORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Gly Gly Gly Tyr His Cys Arg Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

) SEQUENCE DESCRIPTION: SEQ ID NO:42:

y Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

:o Gln Gly Gly
20

:ORMATION FOR SEQ ID NO:43:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

:ly Gly Ile Tyr Lys Cys Leu Met Gly Pro Leu Thr Trp Val Cys Thr
5 10 15

:ro Asp Gly Gly
20

NFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Gly Gly Leu Tyr Ser Cys Leu Met Gly Pro Ile Thr Trp Leu Cys Lys
1 5 10 15

Pro Lys Gly Gly
20

:NFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION: SEQ ID NO:45:

/ Gly Asp Tyr His Cys Arg Met Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

ɔ Leu Gly Gly
20

ORMATION FOR SEQ ID NO:46:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ly Gly Asp Tyr Ser Cys Arg Met Gly Pro Thr Thr Trp Val Cys Thr
5 10 15

ro Pro Gly Gly
20

IFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Gly Gly Asp Tyr Trp Cys Arg Met Gly Pro Ser Thr Trp Glu Cys Asn
1 5 10 15

Ala His Gly Gly
20

FORMATION FOR SEQ ID NO:48:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Gly Gly Lys Tyr Leu Cys Ser Phe Gly Pro Ile Thr Trp Val Cys Ala
1 5 10 15

Arg Tyr Gly Gly
20

NFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Gly Gly Leu Tyr Lys Cys Arg Leu Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:50:

:ly Gly Ser Tyr Thr Cys Arg Phe Gly Pro Glu Thr Trp Val Cys Arg
5 10 15

?ro Asn Gly Gly
20

NFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Gly Gly Ser Tyr Ser Cys Arg Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Pro Gly Gly Gly
20

INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Gly Gly Ser Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Gly Gly Leu Tyr Glu Cys Arg Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Lys Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Gly Val Tyr Ser Cys Arg Met Gly Pro Thr Thr Trp Glu Cys Asn
1 5 10 15

Arg Tyr Val Gly
20

INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Gly Gly Ala Tyr Leu Cys His Met Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gln Gly Gly
20

INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Gly Gly Glu Tyr Ser Cys Arg Met Gly Pro Asn Thr Trp Val Cys Lys
1 5 10 15

Pro Val Gly Gly
20

INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ly Gly Val Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Arg
5 10 15

ro Thr Gly Gly
20

IFORMATION FOR SEQ ID NO:62:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Gly Gly Asp Tyr Asn Cys Arg Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Gly Gly Ser Tyr Leu Cys Arg Phe Gly Pro Thr Thr Trp Leu Cys Ser
1 5 10 15

Ser Ala Gly Gly
20

:) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ly Gly Leu Tyr Leu Cys Arg Met Gly Pro Val Thr Trp Glu Cys Gln
5 10 15

ro Arg Gly Gly
20

FORMATION FOR SEQ ID NO:59:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:59:

3ly Gly Leu Tyr Thr Cys Pro Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Leu Pro Gly Gly
20

NFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Val Thr Trp Val Cys Thr
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

ly Gly Trp Val Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gly
5 10 15

al His Gly Gly
20

NFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Gly Gly Gln Leu Leu Cys Gly Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Trp Val Gly Gly
20

INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Gly Gly Lys Tyr Ser Cys Phe Met Gly Pro Thr Thr Trp Val Cys Ser
1 5 10 15

Pro Val Gly Arg Gly Val
20

INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION: SEQ ID NO:77:

/ Gly Leu Tyr Leu Cys Arg Met Gly Pro Gln Thr Trp Met Cys Gln
5 10 15

ɔ Gly Gly Gly
20

ORMATION FOR SEQ ID NO:78:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:78:

ly Gly Asp Tyr Val Cys Arg Met Gly Pro Met Thr Trp Val Cys Ala
5 10 15

ro Tyr Gly Arg
20

IFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Gly Gly Trp Tyr Ser Cys Leu Met Gly Pro Met Thr Trp Val Cys Lys
1 5 10 15

Ala His Arg Gly
20

FORMATION FOR SEQ ID NO:80:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

ci) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Gly Gly Lys Tyr Tyr Cys Trp Met Gly Pro Met Thr Trp Val Cys Ser
1 5 10 15

Pro Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Gly Gly Tyr Val Met Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Ile Pro Gly Gly
20

INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:82:

;ly Ser Cys Leu Gln Cys Cys Ile Gly Pro Ile Thr Trp Val Cys Arg
5 10 15

Iis Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

Gly Gly Asn Tyr Phe Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gln
1 5 10 15

Arg Ser Val Gly
20

:NFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

Gly Gly Glu Tyr Ile Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Lys
1 5 10 15

Arg Thr Gly Gly
20

INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Gly Gly Leu Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Tyr Met Ala Gly
20

NFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Gly Gly Gln Tyr Leu Cys Thr Phe Gly Pro Ile Thr Trp Leu Cys Arg
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Gly Gly Tyr Thr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Ala His Gly Gly
20

INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Gly Gly Thr Tyr Lys Cys Trp Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Val Gly Gly
20

INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

.) SEQUENCE DESCRIPTION: SEQ ID NO:90:

:y Gly Asn Tyr Tyr Cys Arg Phe Gly Pro Ile Thr Phe Glu Cys His
5 10 15

ro Thr Gly Gly
20

FORMATION FOR SEQ ID NO:91:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:91:

3ly Gly Glu Tyr Leu Cys Arg Met Gly Pro Asn Thr Trp Val Cys Thr
1 5 10 15

Pro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

:xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Gly Gly Trp Val Tyr Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Thr Asn Gly Gly
20

NFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Gly Gly Met Tyr Tyr Cys Arg Met Gly Pro Met Thr Trp Val Cys Lys
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Gly Gly Thr Thr Gln Cys Trp Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Ala Arg Gly Gly
20

FORMATION FOR SEQ ID NO:72:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:72:

ly Gly Pro Tyr His Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gly
5 10 15

ro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Gly Gly Glu Tyr Arg Cys Arg Met Gly Pro Ile Ser Trp Val Cys Ser
1 5 10 15

Pro Gln Gly Gly
20

INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

) SEQUENCE DESCRIPTION: SEQ ID NO:74:

y Gly Asn Tyr Thr Cys Arg Phe Gly Pro Leu Thr Trp Glu Cys Thr
5 10 15

o Gln Gly Gly Gly Ala
20

'ORMATION FOR SEQ ID NO:75:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:75:

ly Gly Ser Trp Asp Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Lys
5 10 15

rp Ser Gly Gly
20

IFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

WE CLAIM:

1. A. peptide dimer comprising two monomeric peptides of 10 to about 40 amino acids in length that bind to EPO receptor, each monomeric peptide comprising a sequence of amino acids $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein X_6 is selected from any of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

2. The peptide dimer of Claim 1 wherein each of said monomeric peptides comprise a sequence of amino acids $YX_2X_3X_4X_5GPX_6TWX_7X_8$, (SEQ ID NO: 2) wherein each of X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

3. The peptide dimer of Claim 2 wherein each of said monomeric peptides comprise a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

4. The peptide dimer of Claim 3 wherein X_4 is R or H; X_5 is F or M; X_6 is I, L, T, M or V; X_7 is D or V; X_9 is G, K, L, Q, R, S, or T; and X_{10} is A, G, P, R, or Y.

5. The peptide dimer of Claim 4 wherein X_1 is D, E, L, N, S, T or V; X_2 is A, H, K, L, M, S, or T; X_4 is R or H; X_9 is K, R, S, or T; and X_{10} is P.

6. The peptide dimer of Claim 1 wherein said monomeric peptides are

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18)

7. A pharmaceutical composition comprising at least one peptide dimer of any one of Claims 1-6.

8. A method for treating a patient having a disorder characterized by a deficiency of EPO or low or defective red blood cell population comprising administering to said patient a therapeutically effective amount of at least one peptide dimer of any one of Claims 1-6.

9. The peptide dimer of any one of Claims 1-6 wherein said dimer is formed by a polyethylene glycol linker through a covalent bond.

10. The peptide dimer of any one of Claims 1-6 wherein said monomeric peptide units are dimerized on activated benodiazepins, oxazalones, azalactones, aminimides or diketopiperazine.

11. The peptide dimer of Claim 9 wherein said monomeric peptides are covalently bound N-terminus to N-terminus.

12. The peptide dimer of Claim 10 wherein said monomeric peptides are covalently bound N-terminus to N-terminus.

13. The peptide dimer of Claim 9 wherein said monomeric peptides are covalently bound N-terminus to C-terminus.

14. The peptide of Claim 10 wherein said monomeric peptides are covalently bound N-terminus to C-terminus.

15. A method of improving the bioactivity of a cell surface receptor comprising dimerizing a monomeric agonist of said cell surface receptor and contacting said formed dimer with said cell surface receptor to effect said improved biological activity.

16. A method of activating a cell surface receptor to induce biological activity of said cell surface receptor comprising dimerizing a monomeric agonist of said cell surface receptor and contacting said formed dimer with said receptor thereby inducing said biological activity.

17. The method of Claim 15 or 16 wherein said cell surface receptor is contacted with said dimer in vitro or in vivo.

18. The method of Claim 15 or 16 wherein said cell surface receptor is EPO-R.

19. The method of Claim 15 or 16 wherein said cell surface agonist is a GH agonist, PDGF agonist, ECF agonist, G-CSF agonist, TPO agonist, VEGF agonist, FGF

agonist, insulin agonist, IL-3 agonist, IL-5 agonist, IL-6 agonist or IL-2 agonist.

20. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $YX_2X_3X_4X_5GPX_6TWX_7X_8$, (SEQ ID NO: 2) wherein each of X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

21. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

22. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1Y_1X_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2 and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R or H; X_5 is F or M; X_6 is I, L, T, M or V; X_7 is D or V; X_9 is G, K, L, Q, R, S, or T; and X_{10} is A, G, P, R, or Y.

23. The method of Claims 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein X_1 is D, E, L, N, S, T or V; X_2 is A, H, K, L, M, S, or T; X_3 is C; X_4 is R or H; X_5 is M, F or I; X_6 and X_{11} are independently any one of the 20 genetically coded L-

amino acids; X_7 is D, E, I, L or V; X_8 is C; X_9 is K, R, S, or T; and X_{10} is P.

24. The method of Claim 15 or 16 wherein said agonist is selected from the group consisting of:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16); and
YCHFGPLTWVC (SEQ ID NO: 17).

25. The method of Claim 15 or 16 wherein said peptide dimers are formed with a polyethylene glycol linker through a covalent bond.

26. A method of preparing a cell surface receptor agonist comprising dimerizing a cell surface antagonist.

27. The method of Claim 26 wherein said cell surface antagonist receptor is a GH antagonist, PDGF antagonist, EGF antagonist, G-CSF antagonist, EGF antagonist, GM-CSF antagonist, TPO antagonist, VEGF antagonist, FGF antagonist, insulin antagonist, IL-3 antagonist, IL-5 antagonist, IL-6 antagonist, or an IL-2 antagonist.

28. The method of Claim 26 wherein said cell surface receptor antagonist is a EPO-R antagonist.

29. The method of Claim 28 wherein said antagonist comprises a sequence of amino acids $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_6 is selected from any of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; X_8 is C; X_2 is selected from any of the 20 genetically coded L-amino acids, n is O or 1 and A is any of the 20 genetically coded L-amino acids except Y (tyrosine).

30. The method of Claim 21 where said antagonist is SCHFGPLTWVCK (SEQ ID NO: 18).

Reverse Phase Analysis of SPA2 Reaction with SEQ. I.D. NO. 8

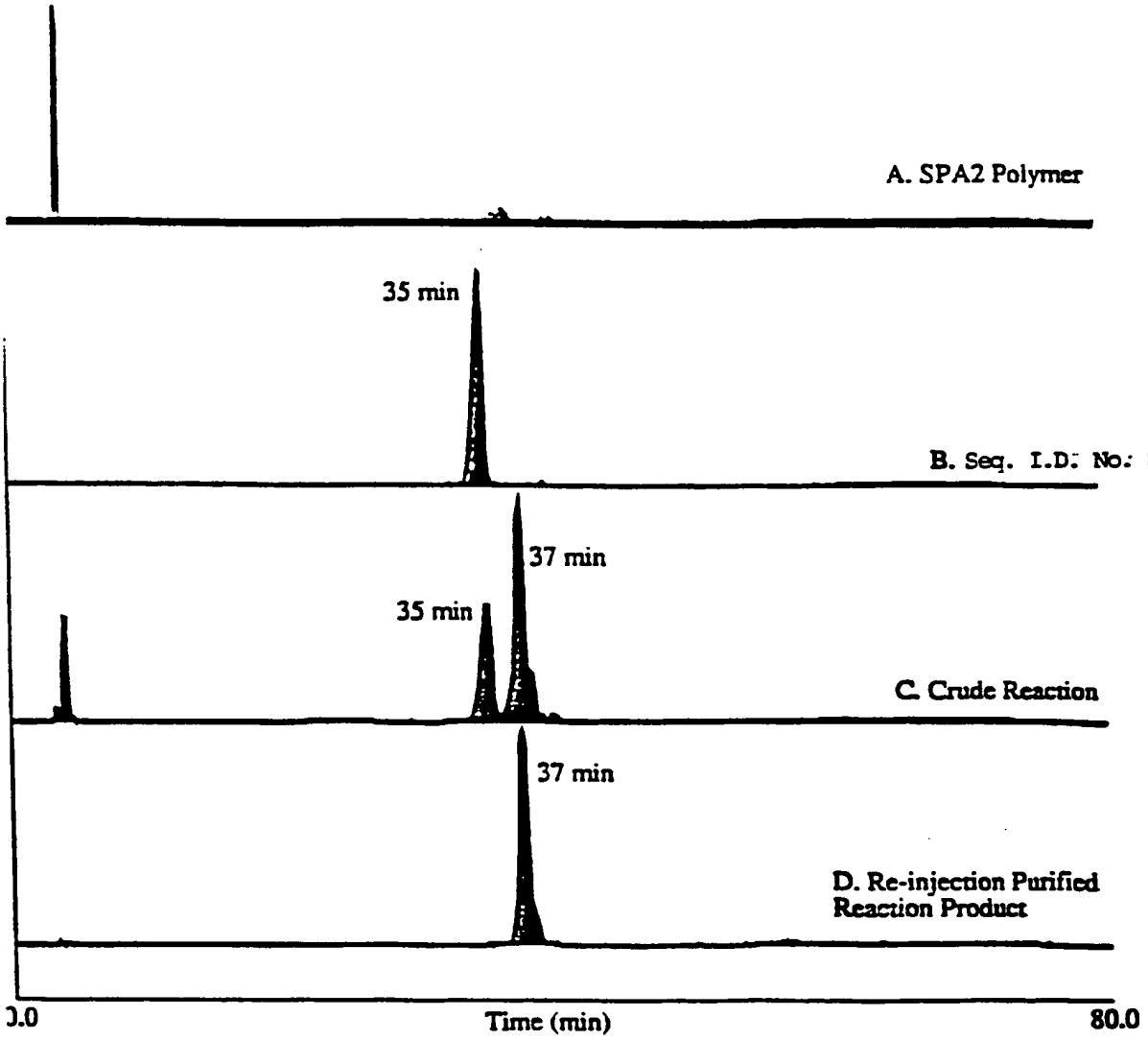


Figure 1

Preparative Reverse Phase Analysis of PEG- Peptide (SEQ. I.D. No. 8) Dimer

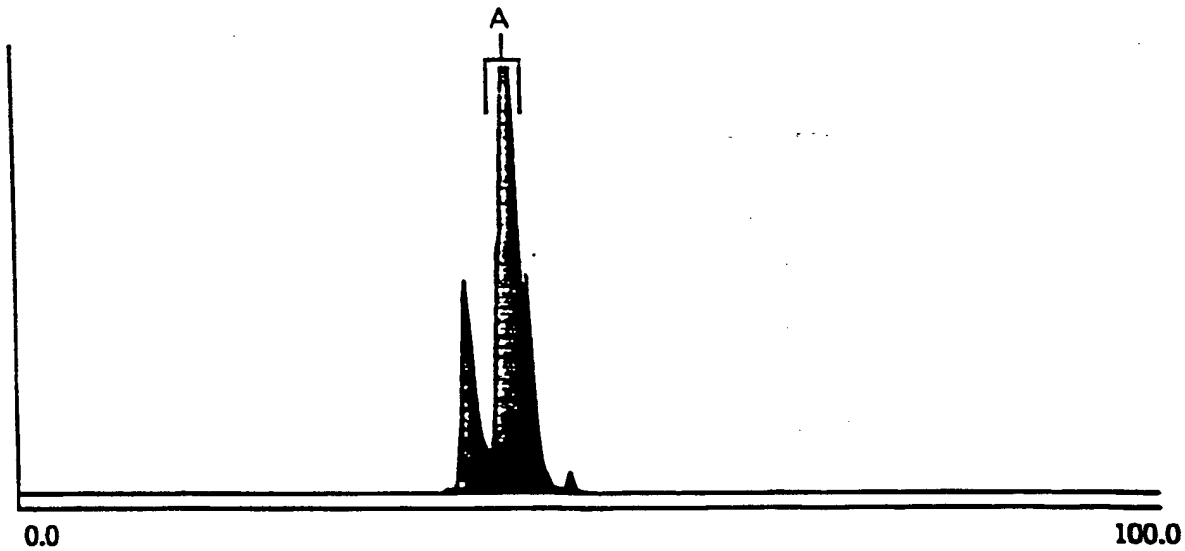


Figure 2

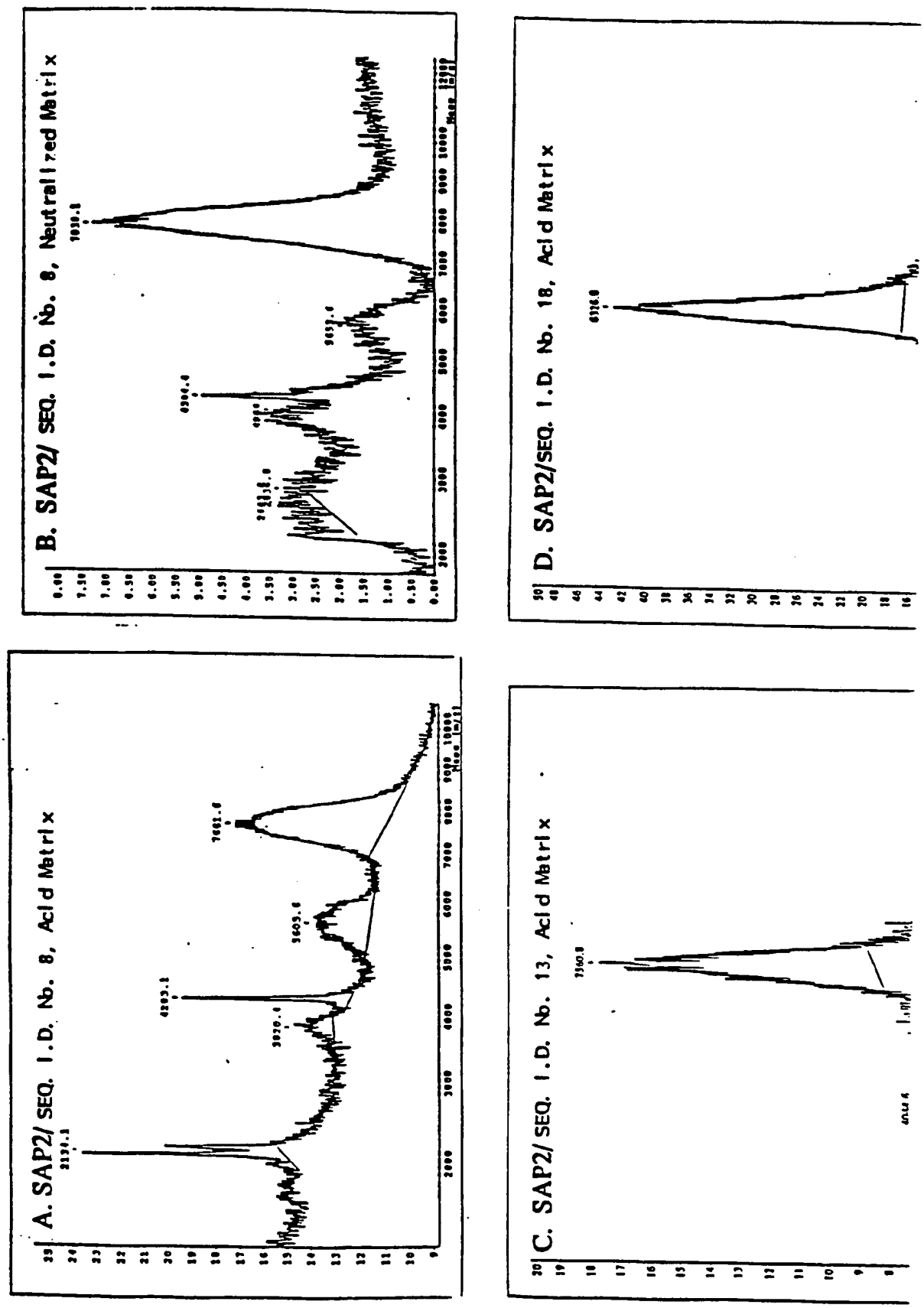
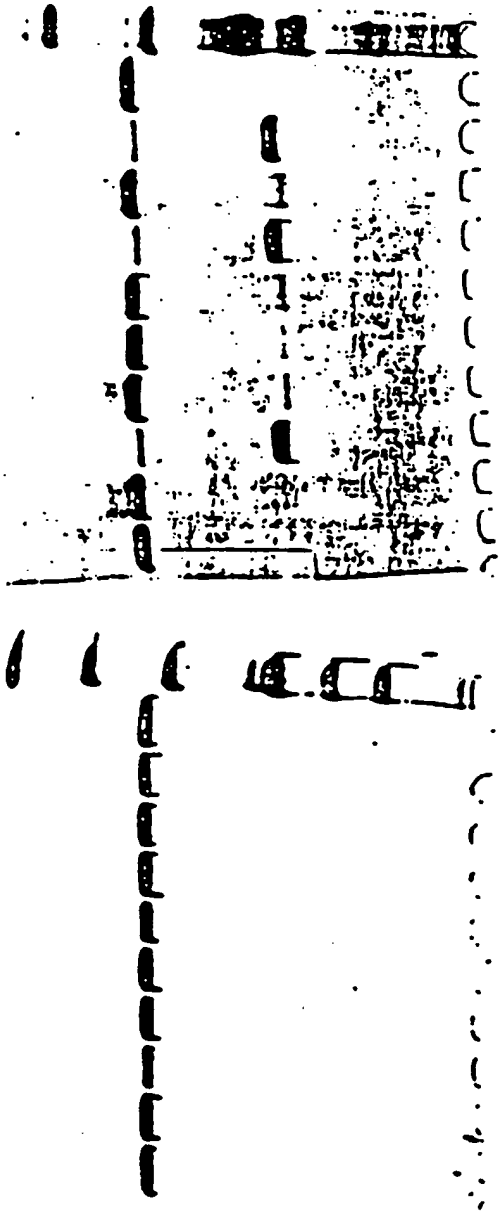

A. SAP2/ SEQ. I.D. No. 8, Acid Matrix
B. SAP2/ SEQ. I.D. No. 8, Neutralized Matrix
C. SAP2/ SEQ. I.D. No. 13, Acid Matrix
D. SAP2/SEQ. I.D. No. 18, Acid Matrix

Figure 3



A. Non Reducing 10-20% SDS-PAGE, B. Reducing 10-20% SDS-PAGE

Lane	1	2	Seq. I.D. Nb.	3	4	5	6	7	8	9	10	11
MWM	EBP	Peptide (μM)		400	400	400	400	400	400	400	400	...
	EBP (μM)			22	22	22	22	22	22	22	22	22
		DPDYB (mM)	1.1	0	1.1	0	1.1	0	1.1	0	1.1	0

Seq. I.D. Nb.	Sequence	IC_{50} (μM)	EPO-ED_{50} (μM)
8	GGTYSCHFGPLTWVCKPQGG	5	0.1
16	YSCHFGPLTWVCK	70	1
18	SCHFGPLTWVCK	90	IA
13	GGTYSCHFGPLTWVCKPQ	8	0.08

Figure 4

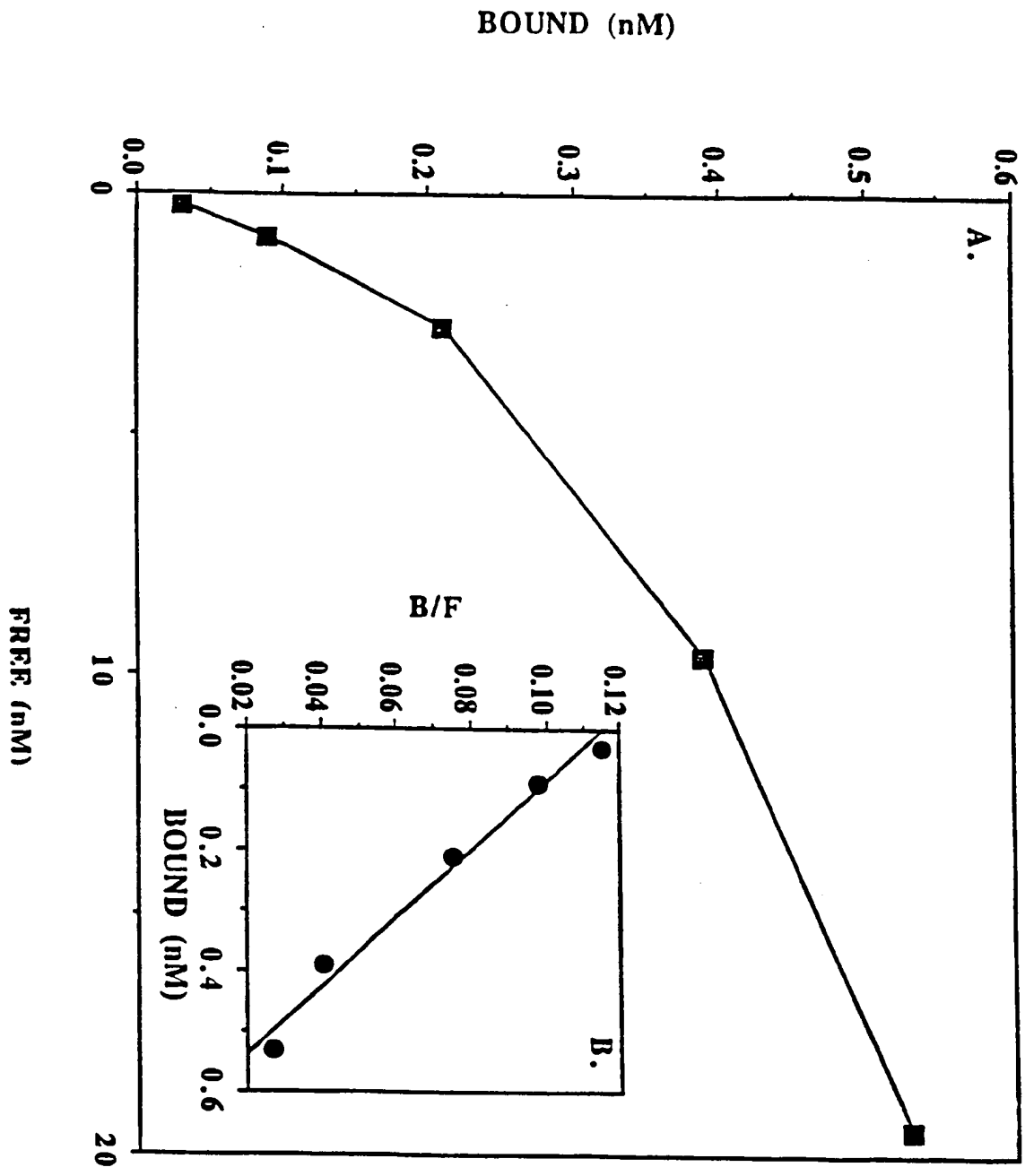
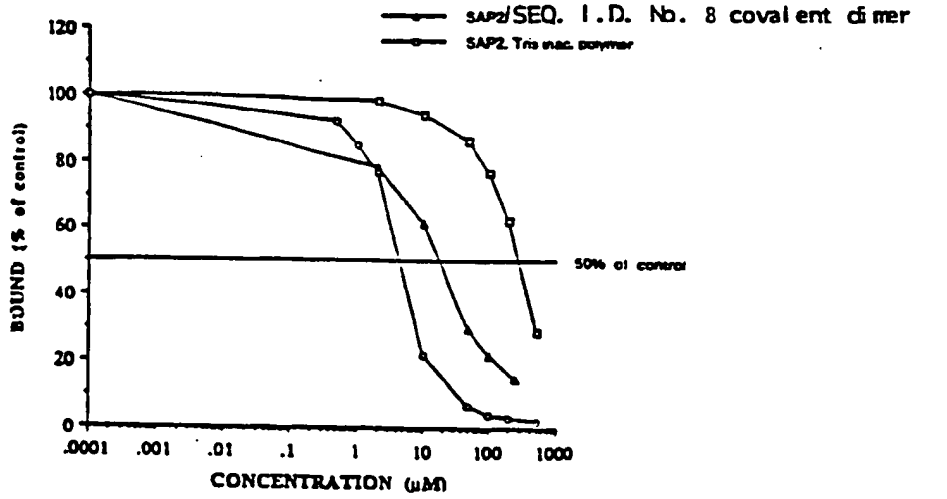
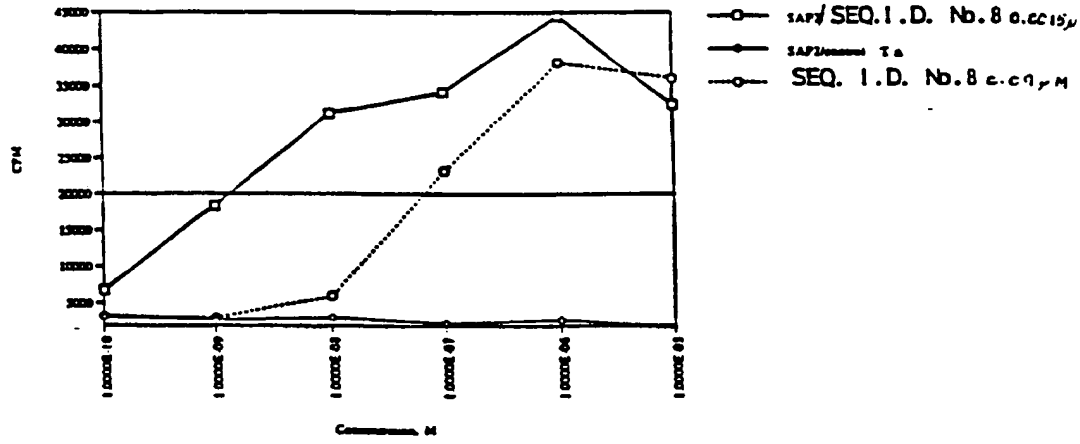

BOUND (nM)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
FREE (nM)
A.
B/F
0.02
0.04
0.06
0.08
0.10
0.12
0.0
0.2
0.4
0.6
BOUND (nM)
B.

Figure 5

A. [125I]EPO Competition Binding Analysis



B. Cell Proliferation, Human EPOR



C. Cell Proliferation, Murine EPOR

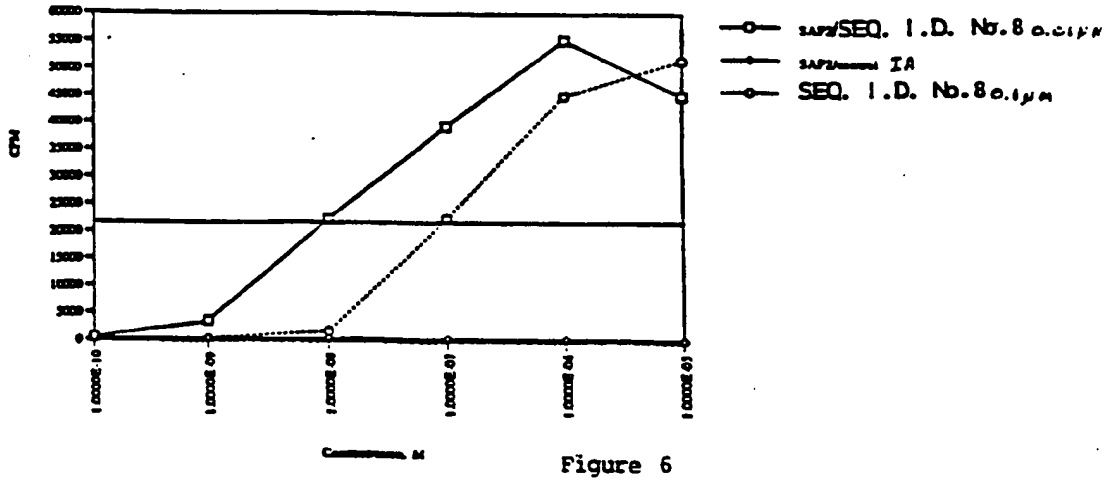


Figure 6

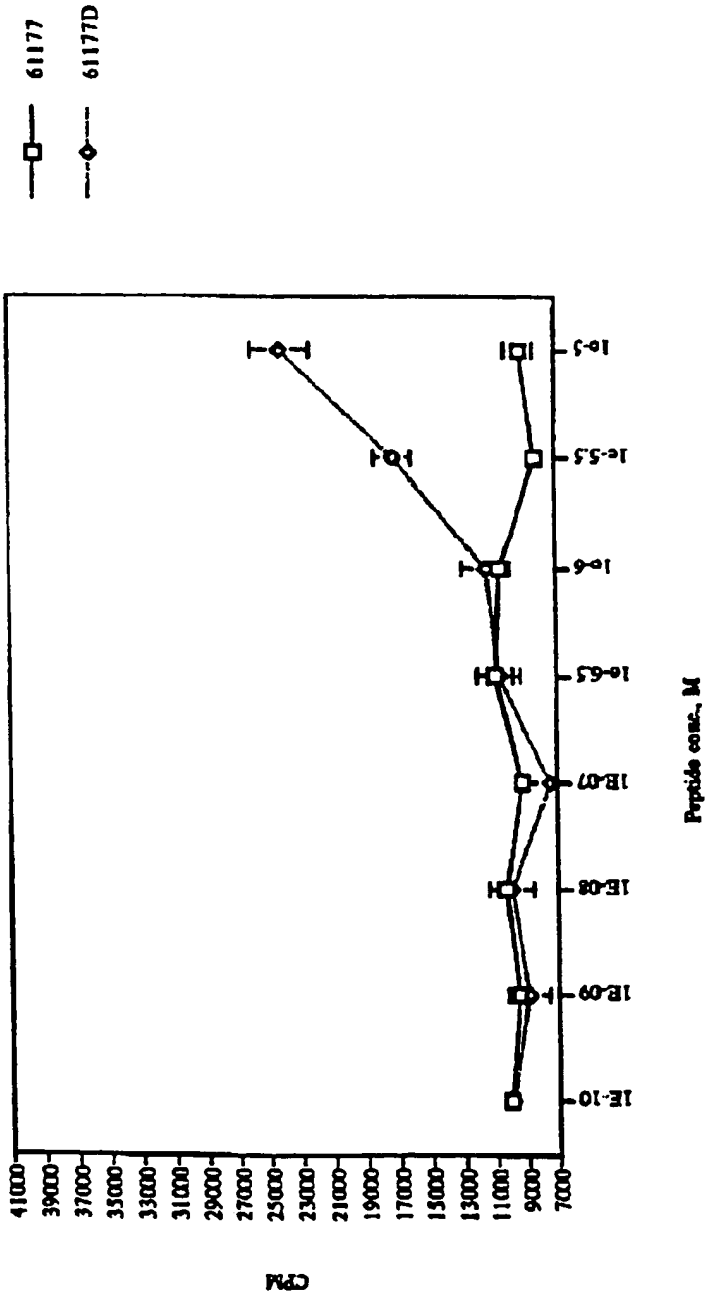


Figure 6
Panel D

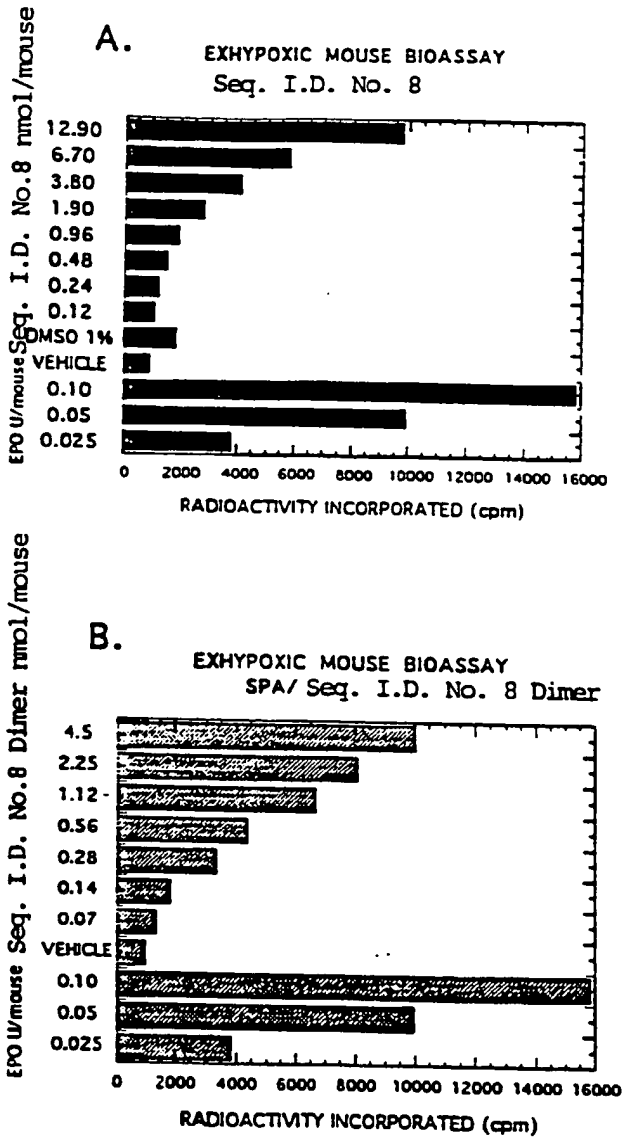

A.
EXHYPOXIC MOUSE BIOASSAY
Seq. I.D. No. 8
EPO U/mouse Seq. I.D. No.8 nmol/mouse
12.90
6.70
3.80
1.90
0.96
0.48
0.24
0.12
DMSO 1%
VEHICLE
0.10
0.05
0.025
0 2000 4000 6000 8000 10000 12000 14000 16000
RADIOACTIVITY INCORPORATED (cpm)
B.
EXHYPOXIC MOUSE BIOASSAY
SPA/ Seq. I.D. No. 8 Dimer
EPO U/mouse Seq. I.D. No.8 Dimer nmol/mouse
4.5
2.25
1.12
0.56
0.28
0.14
0.07
VEHICLE
0.10
0.05
0.025
0 2000 4000 6000 8000 10000 12000 14000 16000
RADIOACTIVITY INCORPORATED (cpm)

Figure 7

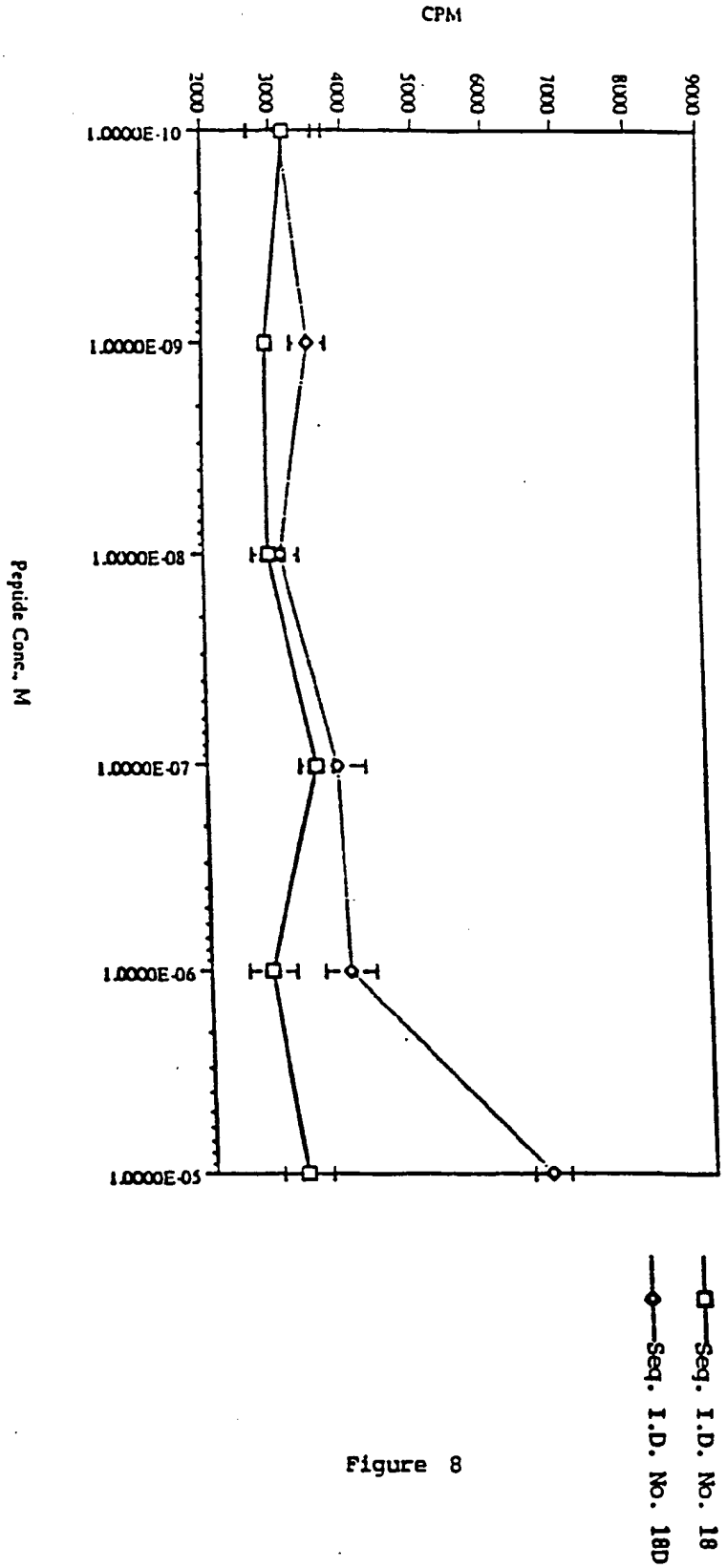

CPM
2000
3000
4000
5000
6000
7000
8000
9000
1.0000E-10
1.0000E-09
1.0000E-08
1.0000E-07
1.0000E-06
1.0000E-05
Peptide Conc., M
Seq. I.D. No. 18
Seq. I.D. No. 18D

Figure 8

FIGURE 9

Page 1 of 3

Ac-GGTYSCHFGPLTWVCKPQGG	SEQ ID NO. 20
[illegible]	SEQ ID NO. 21
GGNYSCRMGPNTWVCGPEGG	SEQ ID NO:22
GGNAWCRMGPITWVCSAEGG	SEQ ID NO:23
GGNYSCRMGPITWVCEPYGG	SEQ ID NO:24
GGEYKCRMGPITWVCKPEGG	SEQ ID NO:25
GGDYTCRMGPNTWICTAYGG	SEQ ID NO:26
GGNYLCRFGPGTWDCTGFRG	SEQ ID NO:27
GGNYVCRMGPITWICTPAGG	SEQ ID NO:28
GGKDVCRMGPITWDCRSTGG	SEQ ID NO:29
GGSYLCRMGPTTWLCTAQRGGG	SEQ ID NO:30
GGNYLCRMGPATWVCGRMGG	SEQ ID NO:31
GGEYKCRMGPLTWVCGYAGG	SEQ ID NO:32
GGDYTCRMGPNTWICTATRG	SEQ ID NO:33
GGVYVCRMGPLTWLCTASGG	SEQ ID NO:34
GGEYSCRMGPNTWVCSPTGG	SEQ ID NO:35
GGEYLCRMGPITWVCERYGG	SEQ ID NO:36
GGNYICRMGPMTWVCTAHGG	SEQ ID NO:37
GGDYLCRMGPATWVCGRMGG	SEQ ID NO:38
GGLYLCRFGPVTWDCGYKGG	SEQ ID NO:39
GGLYSCRMGPITWVCTKAGG	SEQ ID NO:40
GGGYHCRMGPMTWVCRPVGG	SEQ ID NO:41
GGTYSCHFGPLTWVCKPQGG	SEQ ID NO: 42
GGIYKCLMGPLTWVCTPDGG	SEQ ID NO: 43
GGLYSCLMGPITWLCKPKGG	SEQ ID NO: 44
GGDYHCRMGPLTWVCKPLGG	SEQ ID NO: 45
GGDYSCRMGPTTWVCTPPGG	SEQ ID NO: 46
GGDYWCRMGPSTWECNAHGG	SEQ ID NO: 47
GGKYLCSFGPITWVCARYGG	SEQ ID NO: 48
GGLYKCRLGPITWVCSPLGG	SEQ ID NO: 49
GGSYTCRFGPETWVCRPNGG	SEQ ID NO: 50
GGSYSCRMGPITWVCKPGGG	SEQ ID NO: 51
GGSYTCRMGPITWVCLPAGG	SEQ ID NO: 52
GGLYECRMGPMTWVCRPGGG	SEQ ID NO: 53

Figure 9
Page2 of 3

GGDYTCRMGPITWICTKAGG	SEQ ID NO: 54
GGVYSCRMGPTTWECNRYVG	SEQ ID NO: 55
GGAYLCRMGPITWVCRPQGG	SEQ ID NO: 56
GGEYSCRMGPNTWVCKPVGG	SEQ ID NO: 57
GGLYLCRMGPVTWECQPRGG	SEQ ID NO: 58
GGLYTCPMGPITWVCLLPGG	SEQ ID NO: 59
GGLYTCRMGPVTWVCTGAGG	SEQ ID NO: 60
GGVYKCRMGPLTWECRPTGG	SEQ ID NO: 61
GGDYNCRFGPLTWVCKPSGG	SEQ ID NO: 62
GGSYLCRFGPTTWLCSSAGG	SEQ ID NO: 63
GGSYLCRMGPTTWVCTPMGG	SEQ ID NO: 64
GGSYLCRFGPTTWLCTQRGG	SEQ ID NO: 65
GGWVTCRMGPITWVCGVHGG	SEQ ID NO: 66
GGQLLCGIGPITWVCRWVGG	SEQ ID NO: 67
GGKYSCFMGPTTWVCSPVGRGV	SEQ ID NO: 68
GGWVYCRIGPITWVCDTWGG	SEQ ID NO: 69
GGMYYCRMGPMTWVCKGAGG	SEQ ID NO: 70
GGTTQCWIGPITWVCRARGG	SEQ ID NO: 71
GGPYHCRMGPITWVCGPVGG	SEQ ID NO: 72
GGEYRCRMGPISWVCSPQGG	SEQ ID NO: 73
GGNYTCRFGPLTWECTPQGGGA	SEQ ID NO: 74
GGSWDCRIGPITWVCKWSGG	SEQ ID NO: 75
VGNYMCHFGPITWVCRPGGG	SEQ ID NO: 76
GGLYLCRMGPQTWMCQPGGG	SEQ ID NO: 77
GGDYVCRMGPMTWVCAPYGR	SEQ ID NO: 78
GGWYSCLMGPMTWVCKAHRG	SEQ ID NO: 79
GGKYYCWMGPMTWVCSPAGG	SEQ ID NO: 80
GGYVMCRIGPITWVCDIPGG	SEQ ID NO: 81
GSCLQCCIGPITWVCRHAGG	SEQ ID NO: 82
GGNYFCRMGPTTWVCQSVG	SEQ ID NO: 83
GGEYICRMGPLTWECKRTGG	SEQ ID NO: 84
GGLYACRMGPITWVCKYMAG	SEQ ID NO: 85
GGQYLCTFGPITWLCRGAGG	SEQ ID NO: 86
GGVYACRMGPITWVCSPLGG	SEQ ID NO: 87
GGYTTCRMGPITWVCSAHGG	SEQ ID NO: 88
GGTYKCWMGPMTWVCRPVGG	SEQ ID NO: 89
GGNYYCRFGPITFECHPTGG	SEQ ID NO: 90

Figure 9
Page 3 of 3

GGEYLCRMGPHTRVCTPVGG SEQ ID NO: 91
GGLYTCRMGPITRVCLPAGG SEQ ID NO: 92
GGLYTCRMGPITRVCLPAGG SEQ ID NO: 93

CORRECTED VERSION*

PCT

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(54) Title: AGONIST PEPTIDE DIMERS

(57) Abstract

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is idependently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

* (Referred to in PCT Gazette No. 07/1997, Section II)

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AGONIST PEPTIDE DIMERS

FIELD OF THE INVENTION

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO) is a glycoprotein hormon with an approximate molecular weight of 34,000 daltons. The primary role of EPO, which is synthesized in the kidneys of mammals, is to stimulate mitotic cell division and differentiation of erythrocyte precursor cells. As a result, EPO acts to stimulate and to

regulate the production of erythrocytes. Erythrocytes, and the hemoglobin contained therein, play a central role in supplying oxygen to the body. Thus, the stimulation of erythrocyte production is able to increase the oxygen-carrying capacity of the blood.

During normal conditions, EPO is present in very low concentrations in plasma. Under hypoxic conditions, the amount of EPO in the circulation is increased in response to reduced O_2 blood levels. Hypoxia may be caused from various conditions including the loss of large amounts of blood, destruction of red blood cells by over-exposure to radiation or chemotherapeutic agents, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or by various forms of anemia. As the hypoxic condition diminishes, the amount of EPO produced subsequently diminishes.

Because of the essential role of EPO in red blood cell formation, the hormone is useful in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. Recent studies provide a basis for the efficacy of EPO therapy in a variety of disease states, disorders, and states of hematologic irregularity, including: beta-thalassemia (See, Vedovato et al. (1984) Acta. Haematol. 71:211-213); cystic fibrosis (See, Vichinsky et al. (1984) J. Pediatric 105:15-21); pregnancy and menstrual disorders (See, Cotes et al. (1983) Brit. J. Ostet. Gyneacol. 90:304-311); early anemia of prematurity (See, Haga et al. (1983) Acta Pediatr. Scand. 72:827-831); spinal cord injury (See,

Claus-Walker et al. (1984) Arch. Phys. Med. Rehabil. 65:370-374); space flight (See, Dunn et al. (1984) Eur. J. Appl. Physiol. 52:178-182); acute blood loss (See, Miller et al. (1982) Brit. J. Haematol. 52:545-590); aging (See, Udupa et al. (1984) J. Lab. Clin. Med. 103:574-588); various neoplastic disease states accompanied by abnormal erythropoiesis (See, Dainiak et al. (1983) Cancer 5:1101-1106); and renal insufficiency (See, Eschbach et al. (1987) N. Eng. J. Med. 316:73-78).

Although purified, homogenous EPO has been characterized, little is known about the mechanism of EPO-induced erythroblast proliferation and differentiation. The specific interaction of EPO with progenitor cells of immature red blood cells, platelets, and megakaryocytes has not been described. This is due in part, to the small number of surface EPO receptor molecules on normal erythroblasts and on the erythroleukemia cell lines. See Krantz and Goldwasser (1984) Proc. Natl. Acad. Sci. USA, 81:7574-7578; Branch et al. (1987) Blood 69:1782-1785; Mayeux et al. (1987) FEBS Letters 211:229-223; Mufson and Gesner (1987) Blo d 69:1485-1490; Sakaguchi et al. (1987) Biochem. Biophys. Res. Commun. 146:7-12; Sawyer et al. (1987) Proc. Natl. Acad. Sci. USA 84:3690-3694; Sawyer et al. (1987) J. Biol. Chem. 262:5554-5562; and Todokoro et al. (1988) Proc. Natl. Acad. Sci. USA 84:4126-4130. The DNA sequences and encoded peptide sequences for murine and human EPO receptor proteins have been described. See, D'Andrea et al. PCT Patent Publication No. WO 90/08822 (published 1990).

The EPO-receptor (EPO-R) belongs to the class of growth-factor-type receptors which are activated by a ligand-induced protein dimerization. Other hormones and cytokines such as human growth hormone (hGH), granulocyte colony stimulating factor (G-CSF), epidermal growth factor (EGF) and insulin can cross-link two receptors resulting in juxtaposition of two cytoplasmic tails. Many of these dimerization-activated receptors have protein kinase domains within the cytoplasmic tails that phosphorylate the neighboring tail upon dimerization. While some cytoplasmic tails lack intrinsic kinase activity, these function by association with protein kinases. The EPO receptor is of the latter type. In each case, phosphorylation results in the activation of a signaling pathway.

In accordance with the present invention, it has been discovered that the dimerization of peptide agonists and antagonists of dimerization-mediated receptors, such as EPO-R, increase the biological efficacy relative to the biological activity of the 'monomeric' agonists and alters the properties of the antagonists such that, these dimers function as agonists, exhibiting biological activity.

SUMMARY OF THE INVENTION

In a first embodiment, the present invention is directed to peptide dimers which behave as cell-surface receptor agonists, dimers which exhibit binding and signal initiation of growth factor-type receptors. In one embodiment, the present invention provides peptide dimers which behave as EPO agonists. These

dimers have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 residues in length, comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

More preferably, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3), where each amino acid is indicated by standard one letter abbreviation; each X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is

homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

In a more preferred embodiment, both X_3 and X_8 are C and thus, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 4). More preferably, the monomeric peptide unit comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 5), where X_4 can be R or H; X_5 can be F or M; X_6 can be I, L, T, M, or V; X_7 is D or V; X_9 can be G, K, L, Q, R, S, or T; and X_{10} can be A, G, P, R, or Y. In a most preferred embodiment, the monomeric peptide unit of the dimer will comprise a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 6), where X_1 can be D, E, L, N, S, T, or V; X_2 can be A, H, K, L, M, S, or T; X_4 is R or H; X_9 can be K, R, S, or T; and X_{10} is P. Particularly preferred monomeric peptide units of the dimers include:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and

SCHFGPLTWVCK (SEQ ID NO: 18).

Other particularly preferred monomeric peptide units of the present dimers include peptides comprising a core sequence of the formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When (AX_2) is present, i.e. when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to delete the Y, tyrosine residue in SEQ ID NOS. 21 - 93. Such monomeric peptides can also be prepared by substitution of Y in position A in the peptides of Fig. 9.

In accordance with the present invention the monomeric units of the dimers can be the same or different.

In a preferred embodiment polyethylene glycol (PEG) is employed as a linker to form the dimeric peptides of the present invention through a covalent bond.

In another embodiment, the present invention is directed to pharmaceutical compositions comprising at least one dimer peptide of the invention and a pharmaceutical carrier.

In a further embodiment, the present invention provides a method for therapeutically treating a mammal having a condition resulting from a hormone or growth factor deficiency by administration of at least one of the dimer peptides of the present invention.

In a still further embodiment, a method for therapeutically treating a mammal having a condition resulting from a deficiency of EPO or from reduced levels of blood oxygen caused by a decrease in erythrocyte number is provided.

In another embodiment of this invention, a method is provided for preparing agonists of cell-surface receptors wherein agonists of the class of cell-surface or dimerization-mediated receptors are dimeriz d to enhance the in vitro or in vivo biological activity of the cell-surface receptor relative to the monomeric agonists from which the dimer is derived. This method is also directed to the preparation of agonists of such growth-factor-type receptors by dimerizing antagonists of these receptors; the dimerized 'antagonists' thereby exhibit agonist biological activity in vitro and in vivo. In a preferred embodiment, the present method is directed to the preparation of EPO-R dimer agonists from monomeric EPO-R antagonists.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a major peak, with a retention time of 37 minutes, of the dimerized EPO peptide, GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8)

Fig. 2 shows a major peak, with a retention time of 48 minutes, following purification of the dimerized EPO peptide, (SEQ ID NO: 8).

Fig. 3 depicts the MALDI-TOF mass spectral analysis of the dimerized peptides, including peptide (SEQ ID NO: 8), GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13) and SCHFGPLTWVCK (SEQ ID NO: 18).

Fig. 4 shows the SDS-PAGE analysis of DPDPB crosslinking of EPO binding protein (EBP) in the presence and absence of EPO agonist peptides.

Fig. 5 demonstrates equilibrium EPO binding to immobilized EPO binding protein. Panel A represents the equilibrium binding data and Panel B (inset) is the linear transformation (Scatchard) of the data set in Panel A.

Fig. 6 depicts the results of a competitive binding assay run on the EPO agonist peptide (SEQ ID NO: 8) in competitive binding with [^{125}I]EPO to EBP beads (Panel A); and EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing either a human (Panel B) or murine EPO receptor (Panel C).

Fig. 7 is a graphic representation of the results of the exhypoxic mouse bioassay; stimulation of the incorporation of [^{59}Fe] into nascent red blood cells by EPO, peptide (SEQ ID NO: 8) (Panel A) and peptide (SEQ ID NO: 8) dimer (Panel B).

Fig. 8 demonstrates the effect of PEG dimerization of peptide (SEQ ID NO: 18) activity in EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing a human EPO receptor.

Fig. 9 provides the sequences of representative monomeric peptides of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to peptide dimers which behave as cell surface receptor agonists, dimers which exhibit binding and signal initiation of growth-factor-type receptors. Sometimes called cell-surface receptors, growth-factor-type receptors or dimerization-mediated activator-receptors, these are a class of molecules which are understood to be activated by ligand-induced or ligand stabilized dimerization. Agonists of such receptors typically include large polypeptide hormones including the cytokines, insulin and various other growth or differentiation factors. The agonists are understood to induce dimerization of the receptor and thereby effect signal initiation. Such agonists are believed to effectively cross-link two receptors resulting in the repositioning of cytoplasmic tails which may directly or indirectly effect phosphorylation of the cytoplasmic tails and activation of a signaling pathway.

The present invention specifically includes those molecules which behave as agonists of cell-surface receptors when dimerized in accordance with this invention. Such dimer agonists can include 'monomeric' units which exhibit agonist or antagonist activity for the related receptor molecule and may be the same or different. The dimers are preferably peptides but may alternatively be small molecule pharmacophores. These molecules when dimerized exhibit agonist activity of

cell-surface receptors in vitro and in vivo. Such receptors include, for example, EPO, GM-CSF, G-CSF, M-CSF, GH, EGF, PDGF, VEGF, Insulin and FGF. Other receptors which are activated by heterodimerization or multimerization may also be subject to activation by this mechanism including, IL-3, IL-5, IL-6, IL-2 and TPO. The dimers of the present invention have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 amino acid residues in length. In a preferred embodiment, these monomeric peptide units comprise a core sequence of amino acids $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

More preferably, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3), where each amino acid is indicated by standard one letter abbreviation; each X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

In a more preferred embodiment, both X_3 and X_8 will be C and thus, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 4). More preferably, the monomeric peptide unit comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 5), where X_4 can be R or H; X_5 can be F or M; X_6 can be I, L, T, M, or V; X_7 is D or V; X_9 can be G, K, L, Q, R, S, or T; and X_{10} can be A, G, P, R, or Y. In a most preferred embodiment, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TWX_7CX_9X_{10}X_{11}$ (SEQ ID NO: 6), where X_1 can be D, E, L, N, S, T, or V; X_2 can be A, H, K, L, M, S, or T; X_4 is R or H; X_9 can be K, R, S, or T; and X_{10} is P. Particularly preferred monomeric peptide units of the present dimers include:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);

GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18).

The dimer peptides of the present invention exhibit increased biological potency in vitro and in vivo relative to the monomeric agonists from which the dimers are derived. Moreover, cell surface receptor antagonists can be 'converted' to cell surface receptor agonists in accordance with the present invention. Specifically, a cell surface receptor antagonist can be dimerized with PEG or another appropriate linker which permits mutual binding of the monomeric moieties with the receptors. As a result, the dimer exhibits effective binding to the target receptor and behaves as an agonist. Accordingly, the dimers of this invention demonstrate enhanced biological potency in vitro and in vivo relative to their monomeric forms.

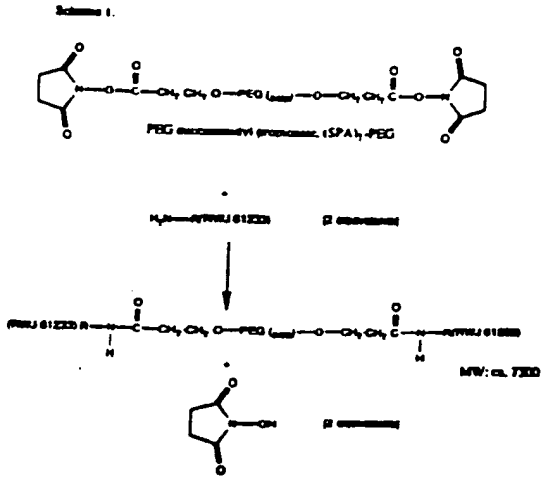
The dimer peptides of the present invention bind to and biologically activate the cell surface receptor or otherwise behave as agonists and are preferably formed by employing polyethylene glycol as a linker between the monomeric peptide units described herein. While other conventional chemical systems can

also be employed to form the dimer peptides of this invention including using other known polymeric compounds, pegylation is preferred.

The linking compounds of the present invention include any molecule which covalently binds the monomeric peptides at an appropriate distance or which otherwise effects dimerization of the particular cell surface receptor thereby initiating biological efficacy.

Starting with an appropriate synthetic peptide, containing a free amino group or other reactive site such as hydroxyls, carboxylic acids or sulfhydryls, the peptide is added in excess to a reaction mixture containing a corresponding reactive polymer. The polymer can be of a repeating nature such as polyethylene glycol, peptides, modified peptides or peptide analogs. Alternatively, the peptide can be dimerized on a small molecule scaffold such as activated benodiazepins, oxazolones, azalactones, aminimides or diketopiperazines. The most readily available linker f variable distance are ones based on linear unbranched polyethylene glycols.

The following is a schematic of a preferred preparatory methodology employing PEG succinimidyl propionate as the linker between the monomer units of the dimer peptides.



Dimerization and especially pegylation in a head-to-head (amino to amino terminus) or head-to-tail (amino to carboxyl terminus) configuration is preferred relative to internal covalent binding of the monomeric peptides. The 'monomer' units of the dimer peptides of the present invention can be the same or different, although the same are preferred.

The monomeric peptides which are used to form the dimers of the present invention can be prepared by classical chemical methods well known in the art. The standard methods include, for example, exclusive solid phase synthesis and recombinant DNA technology. See, e.g. Merrifield (1963) J. Am. Chem. Soc. 85:2149. Solid phase synthesis is typically commenced from the C-terminal end of the peptide using an α-amino protected resin. A suitable starting material can be prepared by attaching the required α-amino acid to a chloromethylated resin (such as BIO-BEADS SX-1, Bio Rad Laboratories, Richmond, CA), a hydroxymethyl resin, (described by Bodonszky et al. (1966) Chem. Ind.

(London) 38:1597) or a benzhydrylamine resin (described by Pietta and Marshall (1970) Chem. Commn. 650).

The α-amino protecting groups are those known to be useful in the art of stepwise synthesis or peptides. Included are acyl type protecting groups (e.g. formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups (e.g. benzyloxycarboyl (Cbz) and substituted Cbz), aliphatic urethane protecting groups (e.g., t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (e.g., benzyl and triphenylmethyl). The preferred X-amino protecting group is Fmoc. The side chain protecting group (typically ethers, esters, trityl, PMC, and the like) remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide.

The side chain protecting groups for Tyr include tetrahydropyranyl, tert-butyl, trityl, benzyl, Cbz, Z-Br-Cbz, and 2,5-dichlorobenzyl. The side chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl, and cyclohexyl. The side chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl, and Cbz. The side chain protecting groups for Thr and Ser are benzyl. The side chain protecting groups for Arg include nitro, Tosy. (Tos), Cbz, adamantyloxycarbonyl mesitoylsulfonyl (Mts), or

Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), Tos, or Boc.

After removal of the α-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. Each protected amino acid is generally reacted in about a 3-fold excess using an appropriate carboxyl group activator such as 2-(1H-benxotriazol-1-yl)-1,1,3,3tetramethyluronium hexafluorophosphate (HBTU) or dicyclohexylcarbodiimide (DCC) in solution of methylene chloride (CH_2CI_2), or dimethyl formamide (DMF) mixtures.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treating the mixture with a reagent such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF). These reagents not only cleave the peptide from the resin, but also cleave all remaining side chain protecting groups. When the chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the desired side chain protected amide or with an alkylamine to give a side chain protected alkylamid or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

These procedures can also be used to synthesize peptides in which amino acids other than the 20 naturally occurring, genetically encoded amino acids are substituted at one, two or more positions of any of the compounds of the invention. For instance, naphthylalanine can be substituted for tryptophan, facilitating synthesis. Other synthetic amino acids that can be substituted into the peptides of the present invention include L-hydroxypropyl, L-3, 4-dehydroxyphenylalanyl, δ amino acids such as L-δ-hydroxylysyl and D-δ-methylalanyl, L-α-methylalanyl, ß amino acids, and isoquinolyl. D-amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides of the present invention.

In another embodiment of the present invention, a method of enhancing the in vitro or in vivo biological potency of a cell surface receptor agonist is provided. This methodology is achieved by dimerizing the receptor agonist with a linker molecule, such as PEG, to form an appropriate spatial relationship between the monomeric peptide units of the dimer and thereby permitting each of the constituents of the dimers to bind to their receptors to achieve enhanced biological potency, i.e., to dimerize and thereby activate the receptors to induce the relevant biological activity of the particular cell-surface receptor, e.g. EPO-R. Biological activity can be measured by the skilled artisan in various in vitro and in vivo assays and as demonstrated in the examples of the present invention.

The peptide or molecule with binding affinity for a given receptor will have increased conformational

flexibility leading to fewer barriers to effective receptor interaction and subsequently receptor activation. This is also indicated for molecules which can bind but not activate a receptor subtype in that such molecules can become more effective inhibitors of ligand binding.

The present invention further provides a method for altering a cell-surface receptor antagonist, a molecule exhibiting receptor binding but no biological activity, to behave as a cell-surface receptor agonist in vitro or in vivo. This method is achieved by dimerizing the antagonist molecule with an appropriate linker molecule such as PEG, other polymerized molecules or a peptide. In a preferred embodiment, an EPO antagonist, i.e. a peptide exhibiting receptor binding but no biological EPO activity can be altered by dimerization to obtain a dimer which behaves as an EPO receptor agonist. Thus, for example, in the case of EPO-R these include the monomeric peptide units of the present dimers comprising a core sequence of general formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein, in (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When X_2 is present, i.e., when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to

delete the Y, tyrosine residue in SEQ ID NOS. 21 - 93. Such monomeric peptides can also be prepared by substitution of Y in the peptides of Fig. 9.

These molecules, demonstrate only binding activity in their 'monomeric' form, but exhibit agonist activity after dimerization with a linking compound such as PEG. Accordingly, the present method comprises identifying a monomeric peptide as herein defined which does not demonstrate biological activity and dimerizing that antagonist in accordance with the present inventi n to obtain a cell-surface-receptor agonist i.e., in dimeric form. Contacting the appropriate cell-surface receptor with the thus formed dimer activates, i.e. dimerizes such receptors and thus induces biological activity of the receptor. Such monomeric units as shown in Fig. 9 can be truncated from the N-terminus such as SCHFGPLTWVCK (SEQ ID NO: 18) to eliminate the tyrosine residue at position A of the formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) or merely substituted with any of the remaining 19 naturally occurring amino acids or with other than a non-naturally occurring aromatic amino acid analog. In accordance with the present invention it has been determined that the tyrosine residue at position A of the foregoing formula is critical to biological activity of the monomer peptide. Deletion or substitution of the tyrosine eliminates biological activity. When dimerized however the entity exhibits enhanced biological activity.

For example, tyrosine (Y) substituted in the formula $YX_2X_3X_4X_5GPX_7X_8$ (SEQ ID NO: 2) by p-

iodohydroxyphenylalanine, p-fluorohydroxyphenylalanine, p-amino-hydroxyphenylalanine act as EPO-R monomer agonists but substitution with threonine or alanine for tyrosine at position Y causes the monomer peptide to act as an EPO-R antagonist. However, when dimerized in accordance with the present invention, such dimers behave as EPO-R agonists. The monomeric peptide units identified at Fig. 9, for example, behave as EPO-R antagonists in the absence of tyrosine at position Y of the formula above. When such antagonists are dimerzied, the dimer behaves as an EPO-R agonist.

In a further embodiment of the present invention, pharmaceutical compositions comprising at least one of the dimers of this invention can be employed to therapeutically treat disorders resulting from deficiencies of biological factors such as EPO, GH, GM-CSF, G-CSF, EGF, PDGF, VEGF, insulin, FGF and the like. These pharmaceutical compositions may contain buffers, salts and other excipients to stabilize the composition or assist in the delivery of the dimerized molecules.

In a preferred embodiment, the present invention provides a method for treating disorders associated with a deficiency of EPO. The method is accomplished by administering at least one of the dimers identified herein for a time and under conditions sufficient to alleviate the symptoms of the disorder, i.e. sufficient to effect dimerization or biological activation of EPO receptors. In the case of EPO such methodology is useful in the treatment of end-stage renal failure/dialysis; anemia, especially associated

with AIDS or chronic inflammatory diseases such as rheumatoid arthritis and chronic bowel inflammation; auto-immune disease; and for boosting the red blood cell count of patient when necessary, e.g. prior to surgery or as pretreatment to transfusion. The dimers of the present invention which behave as EPO agonists can be used to activate megakaryocytes.

Since EPO has been shown to have a mitogenic and chemotactic effect on vascular endothelial cells as well as an effect on central cholinergic neurons (see, e.g., Amagnostou et al. (1990) Proc. Natl. Acad. Sci. USA 87:597805982 and Konishi et al. (1993) Brain Res. 609:29-35), the compounds of this invention can also be used to treat a variety of vascular disorders, such as promoting wound healing, growth of collateral coronary blood vessels (such as those that may occur after myocardial infarction), trauma, and post vascular graft treatment, and a variety of neurological disorders, generally characterized by low absolute levels of acetyl choline or low relative levels of acetyl choline as compared to other neuroactive substances e.g., neurotransmitters.

Accordingly, the present invention includes pharmaceutical compositions comprising, as an active ingredient, at least one of the peptide dimers of the present invention in association with a pharmaceutical carrier or diluent. The dimers of this invention can be administered by oral, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), transdermal (either passively or using iontophoresis or electroporation) or transmucosal

(nasal, vaginal, rectal, or sublingual) routes of administration in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pill, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as it normal practice, additional substances other than inert diluents, e.g., lubricating, agents such as magnesium stearate. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering, agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring and perfuming agents.

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting,

emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

Compositions for rectal or vaginal administration are preferably suppositories which may contain, in addition to the active substance, excipients such as cocoa butter or a suppository wax. Compositions for nasal or sublingual administration are also prepared with standard excipients well known in the art.

The dosage of active ingredient in the compositions of this invention may be varied; however, it is necessary that the amount of the active ingredient shall be such that a suitable dosage form is obtained. The selected dosage depends upon the desired therapeutic effect, on the route of administration, and on the duration of the treatment desired. Generally dosage levels of between 0.001 to 10 mg/kg of body weight daily are administered to mammals.

As can be appreciated from the disclosure above, the present invention has a wide variety of applications. Accordingly, the following examples are offered by way of illustration, not by way of limitation.

EXAMPLE 1

SDS-PAGE gels (10-20% gradient SDS-PAG plates, 84 x 70 x 1.0 mm, Integrated Separation Systems, Natick, MA) were stained with Coomasie Brilliant Blue R-250 (BioRad). A commercial preparation of activated difunctional polyethylene glycol (PEG-succinimidyl-propionate, SPA2, MW ca. 3400) was purchased from Shearwater Polymers, Huntsville, AL as was the monofunctional reagent, methoxy-PEG-succinimidyl-proprionate, MW ca 5000. Peptide (SEQ ID NO: 8) and all other peptides were obtained from the Peptide Synthesis Facility RWJ-PRI, La Jolla, CA or Quality Controlled Biochemical, Hopkinton MA. These peptides were cyclized via oxidation of their intramolecular cysteines, amidated at the C-terminus and mass confirmed by FAB-MS. All were Ellman Reaction negative. Tris base was obtained from BioRad, Hercules, CA. (DPDPB) and trifluoracetic acid (HPLC grade) were obtained from Pierce Chemical Co., Rockford IL.

Mono-PEG conjugation of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8)

This example describes the preparation of mono-PEG conjugates of peptide (SEQ ID NO: 8), using the monofunctional amine reactive polymer analog m-SPA-PEG to be used as a control in experiments described herein. The reaction was carried out with polymer in excess (ca. 3 fold) by resuspending 142.5 mg (0.0286 mmol, MW ca. 5000) of polymer in 4 ml PBS at pH 7.5 and adding 20 mg peptide (SEQ ID NO: 8) (0.0095 mmol, MW 2092) dissolved

in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. The reaction was subsequently adjusted to a final concentration 50 mM Tris by the addition of 1 M tris-HCl at pH 7.5. The reaction mixture was incubated on ice for one hour. Analytical HPLC suggested that there were two main reaction products of essentially equivalent magnitude which were not baseline resolved. Preparative HPLC (using the flatter gradient system described in Example 8) and conservative cuts resulted in collection of two product peaks eluting at ca 44 and 47 minutes. After lyophilization, 24.8 mg, 16.5 mg of each species was recovered, respectively. Mass spectral analysis of these two species demonstrated centroid masses of 7092 (peak 1) and 12036 (peak 2) indicating the coupling of one or two PEG molecules, respectively, to the peptide (Table I).

Tris inactivated polymer. Tris inactivated polymer was formed by incubation of 5 mM SPA2 polymer dissolved in PBS (Gibco, Gaithersburg, MD) with 50 mM tris-HCl, pH 7.5 added and used without further purification.

TABLE 1

Recovery Yield of Peptide Conjugation Reaction and Apparent Molecular Mass of Product

I.D. No.	Sequence	Mass	Conjugation Reagent	Main Product Mass (centroid m/z)	Yield (% of theoretical)
8	GGTYSCHFGPLTWVCKPQGG	2092	SPA2-PEG (MW ca. 3400)	7834	69
			m-SPA-PEG (MW ca. 5000)	7092 (peak 1)	-
				12036 (peak 2)	-
13	GGTYSCHFGPLTWVCKPQ	1978	SPA2-PEG	7560	54
20	Ac-GGTYSCHFGPLTWVCKPQGG	2133	SPA2-PEG	7862	30
14	GGLYACHMGPMTWVCQPLRG	2177	SPA2-PEG	7872	37
18	SCHFGPLTWVCK	1375	SPA2-PEG	6326	45

EXAMPLE 2

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8) (lot #1)

Examples 2 - 7 describe the dimerization of various peptides described by the present invention. The modification of peptide (SEQ ID NO: 8) was carried out by resuspending 25 mg (0.0071 mmol) of polymer in 4 ml PBS at pH 7.5, and adding a 3 fold molar excess of peptide (SEQ ID NO: 8) (0.0213 mmol, 44.5 mg, MW 2092) dissolved in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice. After 3 hours of incubation, an additional 7.5 mg (0.0036 mmol) of lyophilized peptide was added, resulting in a final ratio of 3.5 moles of peptide per each mole of SPA2. The mixture was incubated an additional 17 hours on ice. The reaction mixture was adjusted to a final concentration of 50 mM Tris by the addition of 1 M tris-HCl of pH 7.5 and incubated on ice for 1 hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. After preparative HPLC and lyophilization, 38 mg of PEG dimer was recovered. The theoretical yield for this experiment was 55 mg based n a calculated mass of 7600 mg/mmol for a yield of 69% (Table I).

EXAMPLE 3

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8) (lot #2)

The modification of peptide (SEQ ID NO: 8) was carried out by resuspending 25 mg (0.0071 mmol) of polymer in 4 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 8) (0.0213 mmol, 45.8 mg, MW 2092) dissolved in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 22 hours. At that time, the reaction was adjusted to a final concentration of 50 mM Tris by the addition of 1 M tris-HCl, pH 7.5. The reaction mixture was incubated on ice for 1 hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. After preparative HPLC and lyophilization, 37 mg of PEG dimer was recovered. The theoretical yield for this experiment was 55 mg based on a calculated mass of 7600 mg/mmol for a yield of 68% (Table I).

EXAMPLE 4

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13)

The modification of peptide (SEQ ID NO: 13) was carried out by resuspending 11.2 mg (0.0033 mmol) of polymer in 2.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 13) (0.010 mmol, 20 mg, MW 1978) dissolved in 0.25 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. At that time, 0.25 ml of 1 M tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for one hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. The main preparative reaction product peak eluted at ca 43 minutes. After preparative HPLC and lyophilization, 13.3 mg of PEG dimer was recovered. The theoretical yield for this experiment was 24.42 mg based on a calculated mass of 7400 mg/mmol for a yield of 54% (Table I).

EXAMPLE 5

PEG dimerization of peptide Ac-GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 20)

The modification of peptide (SEQ ID NO: 20) was carried out by resuspending 10.5 mg (0.0031 mmol) of polymer in 2.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 20) (0.0094 mmol, 20 mg, MW 2133) dissolved in 0.25 ml of 0.1% trifluoroacetic acid and the mixture incubated at 4°C for 28 hours. At that time, the reaction as monitored by HPLC was estimated to be approximately 30% complete, the temperature was shifted to ambient and an additional 27 hour incubation provided no net increase in product. Because of possible hydrolysis of the reactive polymer, an additional 5 mg of polymer was added and the incubation was continued for an additional 16 hours. At that time, 0.25 ml of 1 M tris-HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for an additional 1 hour. The sample was subjected to analytical and preparative HPLC using a flatter gradient system as described in Example 8. The main preparative reaction product peak eluting at ca 48 minutes. After preparative HPLC and lyophilization, 10.4 mg of PEG dimer was recovered. The theoretical yield for this experiment was 34.4 mg based on a calculated mass of 7650 mg/mmol for a yield of 30% (Table I).

EXAMPLE 6

PEG dimerization of peptide (SEQ ID NO: 14)

The modification of peptide (SEQ ID NO: 14) was carried out by resuspending 2.6 mg (0.00076 mmol) of polymer in 3.0 ml PBS at pH 7.5 and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 14) (0.00229 mmol, 5 mg, MW 2177) dissolved in 0.1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 26 hours. At that time, 0.25 ml of 1 M-tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for 1 hour. The sample was subjected to analytical and preparative HPLC using the flatter gradient system described in Example 8. The main preparative reaction product peak eluted at ca 46 minutes. After preparative HPLC and lyophilization, 2.2 mg of PEG dimer was recovered. The theoretical yield for this experiment was 24.42 mg based on a calculated mass of 7400 mg/mmol for a yield of 37% (Table I).

EXAMPLE 7

PEG dimerization of peptide (SEQ ID NO: 18)

The modification of peptide (SEQ ID NO: 18) was carried out by resuspending 1.2 mg (0.00036 mmol) of polymer in 0.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of the peptide (0.0011 mmol, 1.5 mg, MW 2177) dissolved in 0.05 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. At that time, 0.1 ml of 1 M tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for 1 hour. The sample was subjected to purification using an analytical HPLC system as described in Example 8. The main reaction product peak eluted at ca 38 minutes. After preparative HPLC and lyophilization, 1 mg of PEG dimer was recovered. The theoretical yield for this experiment was 2.2 mg based on a calculated mass of 6150 mg/mmol for a yield of 45% (Table I).

EXAMPLE 8

Analytical and Preparative HPLC analysis

The accumulation of the dimers described above in Examples 1-7 was monitored by analytical reverse phase HPLC. The analysis was carried out using a Vydac C-18 Protein-Peptide column (0.46 x 25 cm, part no. 218TP54) and a Rainin Gradient HPLC system fitted with a Dynamax dual wavelength detector. At injection, the column was equilibrated in 0.1% TFA in dH_2O and was developed with a 45 minute linear gradient (0-100%) of acetonitrile (ACN) containing 0.1% TFA beginning at 10 minutes after injection. The flow rate was held constant at 1 ml/min. Under these analytical conditions, the SPA2 polymer and tris inactivated polymer did not appear to bind the column while a major reaction product with a retention time (37 minutes) was identified (Figure 1). Peptide (SEQ ID NO: 8) demonstrated a retention time of 35 minutes and the excess peptide utilized in the reaction was clearly distinguished from the nascent reaction products.

The main product reaction product peak was purified by preparative reverse phase HPLC on the same chromatographic system using a Vydac C-18 Protein-Peptide column (2.2 x 25 cm, part no. 218TP15022). Injection of the reaction mix (6 ml) occurred with the column equilibrated at 80:20, H_2O:ACN (both containing 0.1% TFA) at a constant flow rate of 8 ml/min. After a 20 minute wash, the column was developed by application of a linear gradient of 100% ACN/0/1% TFA over 60

minutes. The major product peak eluting at 48 minutes was collected and lyophilized (Figure 2). These elution conditions were subsequently modified to improve the resolution of some conjugation products peptide (SEQ ID NO: 20), mPEG-peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 14) from reaction by products. This was accomplished by application of a flatter linear gradient of 20-80% B over 60 minutes. The variation in retention time due to different peptides and elution condition is described as part of each synthesis example. The materials recovered from the main product peak from each reaction were subsequently analyzed by analytical reverse phase HPLC, MALDI-TOF mass spectrometry, EPO competitive binding potential and for in vitro bioactivity.

The activated PEG used in these experiments has an approximate molecular weight of 3400 and has amine reactive succinimidyl groups on either end of the difunctional linear polymer. This reactivity was employed to couple two equivalents of peptide (SEQ ID NO: 8) (MW= 2092) to the polymer with the concomitant liberation of two succinimidyl moieties resulting in a dimeric product as shown in Scheme I. Peptide (SEQ ID NO: 8) contains two potentially reactive amines, one at the N-terminus of the peptide and one in the side chain of the single lysine within the peptide sequence, so that a number of different connectivities between the two molecules was possible.

MALDI-TOF mass spectral analysis was supportive of the presence of the expected dimeric product (Figure 3) as indicated by a predominant speci s

with a centroid mass of 7661. This data shows that the dimeric product described in the present invention was produced using the methods described herein.

EXAMPLE 9

EBP (EPO Binding Protein) Dimerization

This example demonstrates the interaction of peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 16), peptide (SEQ ID NO: 18) and peptide (SEQ ID NO: 13) with EPO binding protein (EBP) using a bifunctional sulphydryl reactive crosslinker, (1,4-Di-[3'-(2'-pyridyldithio)propionamido]butane DPDPB.

To explore the interaction of peptide (SEQ ID NO: 8) with EBP, a bifunctional sulphydryl reactive crosslinker (DPDPB) was used in an attempt to stabilize a mimetic dependent dimeric structure. Control experiments demonstrated that the crosslinker does not inactivate the EPO binding potential of EBP or the proliferative properties of peptide (SEQ ID NO: 8). As shown in Figure 4, a dimeric EBP product was formed by co-incubation of the peptide, peptide (SEQ ID NO: 8), DPDPB and EBP. This data shows the ability of the peptide (SEQ ID NO: 8) to mediate formation of a solubl receptor dimer. To further explore this question, peptides (SEQ ID NO: 13), (SEQ ID NO: 16) and (SEQ ID NO: 18) were examined for their ability to mediate dimerization. As shown in Figure 4, lanes 7A and 8A, when peptide (SEQ ID NO: 13) was truncated at the carboxyl terminus, it retained good in vitro bioactivity and improved in vivo bioactivity, resulting in a crosslinking signal similar to peptide (SEQ ID NO: 8). However, peptide (SEQ ID NO: 18) did not appear to stabilize the dimerization signal (Figure 4, lanes 9A

and 10A) whereas peptide (SEQ ID NO: 16) (Figure 4, lanes 5A and 6A) gave a strong dimerization band. These two peptides differ by a single N-terminal tyrosine residue and display a similar profile in the in vitro proliferation assay with peptide (SEQ ID NO: 18) being inactive. Peptide (SEQ ID NO: 16) has an ED_{50} of 3 μM on murine receptor cells. Both peptides have similar IC_{50} values indicating that they both retain binding activity. These results demonstrate that EBP dimerization is a property of the EPO peptide series and that the presence of the tyrosine is critical for this activity and that this corresponds to in vitro bioactivity.

EXAMPLE 10

IMMOBILIZED EBP BASED [^{125}I]EPO COMPETITION BINDING ASSAY

This study examined the binding capacity of the EPO PEG dimers to bind EPO receptors.

The extracellular domain of the human erythropoietin receptor (EPO binding protein, EBP) was expressed and overproduced in E. coli. As with many other recombinant eukaryotic proteins produced in E. coli, the protein appeared as an insoluble product in laboratory scale fermentations and was refolded and purified to obtain active protein. EPO binding protein produced by this method contains one free sulfhydryl group which can be modified without effecting the solution phase binding of ligand. In order to immobilize the EPO binding protein for equilibrium binding analysis and for competition binding assay, th EPO binding protein was covalently attached to agarose beads.

The iodoacetyl activation chemistry of Sufolink beads (Pierce Chemical Co, Rockford, IL) is specific for free thiols and assures that the linkage is not easily reversible. EBP-Sulfolink beads were made as follows: SulfoLink gel suspension (10 ml) was mixed with of coupling buffer (40 ml: 50 mM Tris, pH 8.3, 5 mM EDTA) and the gel was allowed to settle. The supernatant was removed and the EPO binding protein (0.3-1 mg/ml in coupling buffer) to be bound was add d directly to the washed beads. The mixture was rocked

gently for 30 minutes at room temperature and the beads were allowed to settle for 1 hour at room temperature. The supernatant was removed and retained. The beads were washed twice with 20 ml of coupling buffer. Th washes were recovered as well. The beads were then treated with 20 ml of 0.05 M cysteine for 30 minutes at room temperature to block unbound sites. Finally, the beads were washed with 50 ml of 1 M NaCl, then with 30 ml of PBS, and resuspended in 20 ml of PBS and stored at 4°C. The amount of EBP which was covalently bound to the beads was determined by comparing the OD_{280} of the original EBP solution to the total OD_{280} recovered in the reaction supernatant and the two 20 ml washes. Typically, 40-60% of the applied EBP remains associated with the beads.

Binding assays were initiated by the addition of EPO binding protein beads (50 µl) to individual reaction tubes. Total binding was measured in tubes containing 0.3-30 nM [^{125}I]EPO (NEN Research Products, Boston MA, 100 µCi/µg). For determination of non-specific binding, unlabelled EPO was added at a level of 1000 fold in excess of the corresponding [^{125}I]EPO concentration. Each reaction volume was brought to 500 µl with binding buffer (PBS/0.2% BSA). The tubes were incubated for five hours (a time period experimentally determined as adequate for the establishment of equilibrium) at room temperature with gentle rocking. After five hours, each reaction mixtur was passed through a 1 ml pipet tip plugged with glass wool. The tubes were washed with 1 ml wash buffer (PBS/ 5% BSA) and this volume as well as 2 additional 1 ml

washes were passed through the pipet tip and collected for determination of the free EPO concentration. Equilibrium binding analysis of the specific association of [^{125}I]EPO with EPO mimetic binding proteins immobilized on these agarose beads indicates a Kd of 5 nM ± 2 based on a linear transformation (Scatchard) of the binding isotherm (Figure 5).

Competitive binding analysis assays of candidate peptides and dimer peptides were performed as outlined below. Individual peptides were dissolved in DMSO to prepare a stock solution 1 mM. Dimer peptides were contained within PBS at a concentration of 5 mM. All reaction tubes (in duplicate) contained 50 μL of EBP beads, 0.5 nM [^{125}I]EPO and 0-500 μM peptide in a total of 500 μL binding buffer.

The final concentration of DMSO was adjusted to 2.5% in all peptide assay tubes. At this concentration DMSO has no detectable effect since an examination of the sensitivity of the assay to DMSO demonstrated that concentrations of up to 25% DMSO (V/V) had no deleterious effect on binding. Non-specific binding was measured in each individual assay by inclusion of tubes containing a large excess of unlabelled EPO (1000 nM). Initial assay points with no added peptide were included in each assay to determine total binding. Binding mixtures were incubated overnight at room temperature with gentle rocking. The beads were then collected using Micro-columns (Isolab, Inc.) and washed with 3 mL of wash buffer. The columns containing the washed beads were placed in 12 x 75 mm glass tubes and bound radioactivity levels determined in

a gamma counter. The amount of bound [^{125}I]EPO was expressed as a percentage of the control (total=100%) binding and plotted versus the peptide concentration after correction for non-specific binding. The IC_{50} was defined as the concentration of the analyte which reduced the binding of [^{125}I]EPO to the EBP beads by 50%. All data are reported as relative to peptide (SEQ ID NO: 8) which demonstrated an IC_{50} of 5 μM.

Competitive binding analysis revealed an IC_{50} of 20 μM for the purified dimer, a value four fold greater than peptide (SEQ ID NO: 8) in the same assay (Figure 5 and Table II). Polymer alone, which was inactivated by treatment with Tris-HCl, demonstrated a detectable competition binding signal but this signal was modest (<10%) at the IC_{50} of the PEG-peptide (SEQ ID NO: 8) dimer.

TABLE II

Table II. Binding and Cell Proliferation Studies

id	Relative Binding[1]	EPO-ED_{50} (μM)[2]	
		murine receptor	truncated human receptor
). No. 8	1	0.1	0.09
ris inact.polymer	60	IA[3]	IA
q. I.D. No. 8 covalent dimer #1	4	0.01 (10X)	0.0015 (60X)
q. I.D. No. 8 covalent dimer #2	3	0.01 (10X)	0.002 (45X)
.D. No. 13	1.6	0.08	0.02
q. I.D. No. 13 covalent dimer	3	0.01 (8X)	0.002 (10X)
.D. No. 20 (N-acetyl)	4	0.03	0.06
q. I.D. No. 20 covalent dimer	12	0.2 (-7X)	0.05
.D. No. 14 (terminal NH_2)	0.6	0.1	0.08
q. I.D. No. 14 covalent dimer	-	0.006 (16X)	0.001 (80X)

[1] Amount required to achieve the half maximal level of EPO dependent proliferation (11pM)
[2] Binding relative to Seq. I.D. No. 8
[3] IA=inactive
[4] Note that all peptides are cyclic and were analyzed as COOH terminal amides (-$CONH_2$)

EXAMPLE 11

EPO DEPENDENT CELL PROLIFERATION ASSAYS

This example shows the improved potency of PEG-EPO peptide dimers to EPO receptors in human and murine cell lines.

Cell line FDC-P1/ER, an EPO-dependent line expressing the murine EPO receptor, was grown and maintained as described previously (Carroll et al. 1991). Also employed was cell line FDC-P1/trER expressing a functional truncated human EPO receptor (missing the C-terminal 40 amino acids). Both cell lines exhibit EPO dependent cellular proliferation. Briefly, cells were maintained in RPMI 1640 media (Gibco/BRL) containing 10% heat-inactivated fetal calf serum and 10 units/ml of recombinant human EPO. For the cellular proliferation assay, FDC-P1/ER or FDC-P1/trER cells were grown to stationary phase, centrifuged, washed with RPMI 1640 media (no EPO), and plated in EPO minus media for 24 hr.

After 24 hours, the cells were counted, resuspended at 800,000 cells/ml and dispensed at 40,000 cells/well. Stock solutions of the peptide dimer (5 mM in PBS) and peptide (10 mM in DMSO) were prepared and dispensed in triplicate to final concentrations of 1 x 10^{-10} M through 1 x 10^{-5} M and adjusted to a final volume of 0.2 ml. Final DMSO concentrations of 0.1% (V/V, maximal) or less were found to have no cellular toxicity or stimulatory effects. A standard EPO dose response curve was generated with each assay series.

After a 42 hr incubation at 37°C (ca. 2 cell doublings) 1μCi/well of [^{3}H] thymidine was added and the incubation continued for 6 hr at which time the cells were harvested and counted to assess [^{3}H]thymidine incorporation as a measure of cell proliferation. Results are expressed as the amount of peptide or dimer peptide necessary to yield one half of the maximal activity obtained with recombinant EPO.

As shown in Figure 5 and Table II, the initial lot of PEG-peptide (SEQ ID NO: 8) dimer demonstrated ED_{50} values of 0.01 μm and 0.0015 μm in EPO responsive cell lines containing the murine or human EPO receptor, respectively. In both cell lines, the parent peptide, peptide (SEQ ID NO: 8), demonstrated an ED_{50} of 0.1 μM, indicating an increase in potency of 10 fold in the murine receptor line and almost 60 fold in the human receptor containing cells. Thus, the dimer was clearly more potent in murine and human lines than the peptides themselves. This was confirmed by generation of a second synthesis lot of PEG-peptide (SEQ ID NO: 8) dimer which resulted in a 10 and 45 fold increase in potency in the murine and human lines, respectively. Polymer alone, which was inactivated by treatment with Tris-HCl, demonstrated no activity in the cell proliferation assay.

A second EPO mimetic peptide, peptide (SEQ ID NO: 13), with the sequence GGTYSCHFGPLTWVCKPQ, was also subjected to a similar PEG dimerization protocol as that described above for peptide (SEQ ID NO: 8). The dimer product of PEG-peptide (SEQ ID NO: 13) is also more active than the unconjugated parent compound (Table II).

Both of these dimer peptides have ultimate ED_{50} values near 0.002μM. In spite of this more modest increase, the experimental evidence clearly indicates that the dimerization of these peptides with PEG results in improved potency.

EXAMPLE 12

To further examine the connectivity of the peptides of the present invention to PEG, peptide molecules, which contained only an internal lysine group were used peptide (SEQ ID NO: 8) analog acetylated at the N-terminus peptide (SEQ ID NO: 20) and a sequence analog peptide (SEQ ID NO: 14) which only had a reactive N-terminal amine were PEG dimerized. In vitro proliferation data of these compounds suggest that potential dimerization through the free amino terminus has the most profound effect on bioactivity giving rise to a species about 80 fold more active than the monomeric parent peptide (SEQ ID NO: 14) dimer. Conjugation through the lysine side chain had no real effect on activity peptide (SEQ ID NO: 20) as did mono-PEG or di-PEG conjugation (Table III). This data indicates that the creation of a head to head dimer (both peptides attached through the N-terminus) using a PEG linker greatly enhances the potency of EPO peptides and approaches a level almost two logs greater than the free parent peptide. Further, this effect was not observed upon simple covalent attachment of linear PEG to peptide (SEQ ID NO: 8) indicating that dimerization is a critical determinant for this increased activity.

TABLE III

Table III. Binding and Cell Proliferation Studies mPEG/

Compound	Relative Binding[3]	EPO-ED_{50} (μM)[1]	
		murine receptor	truncated human receptor
Seq. I.D. No. 8	1	0.1	0.09
mPEG/Seq. I.D. No. 8, peak #1	60	2	0.1
mPEG/Seq. I.D. No. 8, peak #2	>60	1	0.4

[1] Amount required to achieve the half maximal level of EPO dependent proliferation (11pM)
[2] ND=Not determined
[3] IA=inactive
[4] Binding relative to Seq. I.D. No. 8

Note that all peptides are cyclic and were analyzed as COOH terminal amides (-$CONH_2$)

EXAMPLE 13

Polycythemic Exhyposic Mouse Bioassay.

This study demonstrates the ability of peptide (SEQ ID NO: 8)/PEG-dimers to retain in vivo bioactivity. Peptides were assayed for in vivo activity in the polycythemic mouse bioassay adapted from the method described by Cotes and Bangham (1961), Nature 191: 1065-1067. BDF1 mice were allowed to acclimate to ambient conditions for 7-10 days. Body weights were determined for all animals. Low weight animals (<15 grams) were not used. Mice were introduced to hypobaric chambers with a 24 hour conditioning cycle consisting of 0.40% +/- 0.02 atm. for 18 hours followed by 6 hours at ambient pressure for a total of 14 days. Following the 14 day period, mice were placed in ambient pressure for 72 hours prior to dosing. Test samples or recombinant Human Erythropoietin (rHuEPO) standards were diluted in an assay vehicle consisting of Phosphate Buffered Saline (PBS)-0.1% Bovine Serum Albumin (BSA). Peptide sample stock solutions (excluding peptide dimers) were first solubilized in dimethyl sulfoxide (DMSO). Control groups included one group of vehicle alone, and one group of (DMSO) at final concentration of 1%.

Each dose group contained 10 mice. Mice were injected subcutaneously (scruff of neck) with 0.5 ml of the appropriate sample. Forty eight hours following the sample injection, the mice were administered an intraperitoneal injection of 0.2 ml of [^{59}Fe]

(approximately 18.0 milliCuries/milligram, Dupont, NEN) and 0.75 microCuries/Mouse.

Mouse body weights were determined twenty four hours following [^{59}Fe] administration and the mice were sacrificed forty eight hours following the [^{59}Fe] injection. Blood was collected from each animal by cardiac puncture and hematocrits were determined (heparin was used as the anticoagulant). Each blood sample (0.2 ml) was analyzed for [^{59}Fe] incorporation using a Packard gamma counter. Non-responder mice (i.e.,those mice with radioactive incorporation less than the negative control group) were eliminated from the appropriate data set. Mice that had hematocrit values less than 53% were also eliminated.

This assay examined the ability of an exogenously administered compound to induce new red blood cell synthesis, or in other words to function as EPO or an EPO mimetic. The results are derived from sets of 10 animals for each experimental dose. As shown in Figure 7 and Table IV, the data suggests that on a mole equivalent basis, peptide (SEQ ID NO: 8)/PEG-dimer is about 10 fold more active than peptide (SEQ ID NO: 8) monomer. These results are consistent with <u>in vitro</u> results in which increased potency values of 10 fold was observed on murine EPO-R bearing cells.

TABLE IV

Table 4. Erthpoxic Mouse Bioassay Study of PEG Dimer Activity

Compound	Amount required for equivalency to 0.025U EPO (nmol) n=10
Seq. I.D. No. 8	3.8

EXAMPLE 14

This example shows that an inactive truncation analog of peptide (SEQ ID NO: 8), which lacks the critical tyrosine peptide (SEQ ID NO: 18), (SCHFGPLTWVCK), can be converted to an agonist on the human EPO receptor cell line by PEG dimerization. In this experiment, a 10^{-8} M concentration of the parent peptide had no activity above background while the dimeric peptide exhibited a level of proliferation twice as many cpm as background. As shown in Figure 8, the peptide alone (open squares) did not induce proliferation of the EPO responsive cells but upon PEG dimerization (open diamonds) a significant agonist effect was observed. Approximately twice as many cpm incorporated over non-stimulated cells at 10^{-8} M added peptide dimer. The replicate error bars represent the standard deviation of three assay points per concentration of peptide or peptide dimer.

Exhypoxic polycythemic mouse bioassay:

PEG dimer and the monomer parent peptide RWJ 61718 were compared in the exhypoxic mouse bioassay (Table 1). This peptide exhibited an 80 fold increase in in vitro activity upon dimerization. Murine studies (in vivo) of the activity of the dimer compared to the monomer peptide revealed a 250 fold increase in activity of RWJ 61718 upon dimerization. Cellular proliferation studies on this dimer peptide in murine receptor containing cells demonstrated a 16 fold increase over the monomer indicating that the 250 fold increase in vivo might be attributable to other factors such as altered metabolism or prolonged circulatory half-life which occur upon PEG dimerization of the peptide sequence. Thus, in addition to the effect of dimerization alone, the PEG modification has an effect which impacts in vivo activity and may be specific to individual peptide sequences.

Cell associated EPO receptor competition binding assay.

A competitive binding analysis of the ability of selected monomer peptides and their cognate dimer products to compete with radiolabelled EPO binding for cell associated human EPO receptors was performed Erythropoietin Receptor Competition Binding Analysis was preformed as follows. TF-1 cells were maintained in RPMI 1640, 10% fetal calf serum, 1% L-glutamine, 1% penicillin, 0.1% streptomycin and 1 ng/ml of GM-CSF.[125]-EPO was obtained from NEN Research Products. Cells were centrifuged and washed 1 x with binding buffer (RPMI 1640, 5% BSA, 25 mM Hepes, pH 7.5, 0.02% sodium azide) resuspended in binding buffer, and counted using trypan lue as an indicator of viability. Each reaction contained approximately 5 x 10^5 cells, [125]-EPO (0.5 nM), no competitor or peptide or dimer preparation in a final volume of 200 µl. The binding reactions (in duplicate) were incubated overnight at 4°C. Following binding, the tubes were centrifuged at 12,000 rpm for 1 min at 4°C in a

refrigerated centrifuge. The supernatant was removed, the cell pellet resuspended in 100 µl of binding buffer, and the cell suspension layered onto 0.7 ml of bovine calf serum. The tubes were centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was removed, the bottom of the tubes snipped off, and the cell pellets counted in a Micromedic ME plus gamma counter. Non-specific binding was determined by incubating cells with [125]-EPO and a 100-fold excess of non-radioactive EPO. These data demonstrate increases in apparent binding competitive affinity of 3.0 fold, 3.2 fold and 80 fold for peptides RWJ 61233, RWJ 61596 and RWJ 61718, respectively (Table 2). In vivo proliferation studies with these peptides and their dimer derivatives reveal increases in potency of ea. 50 fold, 10 fold and 80 fold, respectively, indicating that the magnitude of increased binding affinity is exceeded by the functional potency of the peptide for two of the three species. Thus, the effect of dimerization and subsequent increase in activity may be one in which the efficiency of receptor stimulation is improved by limiting the lateral diffusion of the receptors away from a binding event. Peptide dimerization therefore likely results in entropic rather than enthalpic gains upon mimetic ligand-receptor association for some peptide dimer sequences.

Unlike the EBP-bead EPO competitive binding assay where peptide dimerization negatively impacted the ability of PEG dimer peptides to compete for receptor binding, the ability to compete for cell associated receptors is increased by dimerization. This may be due to the ability of the cell associated receptor to dimerize while the immobilized EBP monomer likely cannot.

Conversion of inactive to active peptide RWJ 61177 was further studied. An improved and expanded study was performed which confirmed our earlier observation of conversion to an active peptide (Figure 6, Panel D).

TABLE IV. EXHYPOXIC MOUSE BIOASSAY STUDY OF PEG DIMER ACTIVITY

Compound	Amount required for equivalency to 0.025 U EPO (nmol) n=10
RWJ 61233 (seq. ID #8)	3.8
SAP2/61233, covalent dimer	0.28
RWJ 61718 (seq. ID #14)	18
SAP2/61718, covalent dimer	0.07

TABLE V. EPO COMPETITIVE BINDING ANALYSIS OF CELL ASSOCIATED RECEPTORS

Compound	IC_{50} (μM)
RWJ 61233 (seq ID #8)	3.8
SAP2/61233, covalent dimer	0.28
RWJ 61718 (seq ID#14)	18
SAP2/61718, covalent dimer	0.07

SEQUENCE LISTING

NERAL INFORMATION:

i) APPLICANT: Johnson, Dana L
Zivin, Robert A

.i) TITLE OF INVENTION: AGONIST PEPTIDE DIMERS

.i) NUMBER OF SEQUENCES: 93

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(F) ZIP: 11530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/484,135
(B) FILING DATE: 07-JUN-1995
(C) CLASSIFICATION:

.ii) ATTORNEY/AGENT INFORMATION:
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INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

c) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos2) can be R,H,L or W; Xaa(Pos3) can be M,F or I; Xaa(Pos6) can be any one of the 20 L-amino acids or the stereoisomeric D-amino acids; Xaa(Pos9) can be D,E,I,L or V; and Xaa(Pos10) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos1) or Xaa(Pos10) is C or Hoc"

i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

aa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
5 10

FORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Xaa(Pos2) and Xaa(Pos8) can be any on of the 20 L-amino acids; Xaa(Pos3) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos4) can be R,H,L or W; Xaa(Pos5) can be M,F or I; Xaa(Pos11) can be D,E,I,L or V; and Xaa(Pos12) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos3) or Xaa(Pos12) is C or Hoc"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
1 5 10

INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

:) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1), Xaa(Pos 3), Xaa(Pos9), Xaa(Pos14), Xaa(Pos15) and Xaa(Pos16) can be any one of 20 L-amino acids; Xaa(Pos4) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos5) can be R,H,L or W; Xaa(Pos6) can be M,F or I; Xaa(Pos12) can be D,E,I,L or V; and Xaa(Pos13) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos4) or Xaa(Pos13) is C or Hoc"

i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

aa Tyr Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa Xaa Xaa Xaa
5 10 15

:FORMATION FOR SEQ ID NO:4:

:i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Xaa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
1 5 10 15

NFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1), Xaa(Pos3) and Xaa(Pos16) can be any one of the 20 L-amino acids; Xaa(Pos5) can be R or H; Xaa(Pos6) can be F or M; Xaa(Pos9) can be I,L,T,M or V; Xaa(Pos12) can be D or V; Xaa(Pos14) can be G,I,L,Q,R,S or T; Xaa(Pos15) can be A,G,P,R or Y"

i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

aa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
5 10 15

FORMATION FOR SEQ ID NO:6:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

.x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be D,E,L,N,S,T or V; Xaa(Pos3) can be A,H,K,L,M,S or T; Xaa(Pos5) can be R or H; Xaa(Pos6) can be F or M; Xaa(Pos9) can be I,L,T,M or V; Xaa(Pos12) can be D or V; Xaa(Pos14) can be K,R,S or T; Xaa(Pos15) is P and Xaa(Pos16) can be any one of the 20 L-amino acids"

xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Xaa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
1 5 10 15

NFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

'ORMATION FOR SEQ ID NO:8:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ly Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

ro Gln Gly Gly
20

IFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Gly Asp Tyr His Cys Arg Met Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Leu Gly Gly
20

:NFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Gly Asn Tyr Met Ala His Met Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly

INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

:) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ly Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

ro Gln

FORMATION FOR SEQ ID NO:14:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Gly Leu Tyr Ala Cys His Met Gly Pro Met Thr Trp Val Cys Gln
1 5 10 15

Pro Leu Arg Gly
20

NFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Ile Ala Gln Tyr Ile Cys Tyr Met Gly Pro Glu Thr Trp Glu Cys
1 5 10 15

Arg Pro Ser Pro Lys Ala
20

FORMATION FOR SEQ ID NO:16:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

hr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10

FORMATION FOR SEQ ID NO:17:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Tyr Cys His Phe Gly Pro Leu Thr Trp Val Cys
1 5 10

NFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10

INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.) MOLECULE TYPE: peptide

c) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be any one of the 20 L-amino acids; except that Xaa(Pos1) may or may not be Y and Xaa(Pos1) may be any non-naturally occurring aromatic acid analog when Xaa(Pos1) is Y. Xaa(Pos2) and Xaa(Pos8) can be any one of the 20 L-amino acids; Xaa(Pos3) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos4) can be R,H,L or W; Xaa(Pos5) can be M,F or I; Xaa(Pos11) can be D,E,I,L or V and Xaa(Pos12) can be C,A,α-amino-γ-bromobutyric acid or Hoc provided that either Xaa(Pos3) or Xaa(Pos12) is C or Hoc"

:i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

:aa Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
1 5 10

NFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Gln Gly Gly
20

INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ly Gly Thr Tyr Arg Cys Ser Met Gly Pro Met Thr Trp Val Cys Leu
5 10 15

ro Met Gly Gly
20

FORMATION FOR SEQ ID NO:22:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Gly Met Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Gly
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Gly Trp Ala Trp Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Ala His Gly Gly
20

FORMATION FOR SEQ ID NO:24:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

ci) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gly Gly Met Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Ile
1 5 10 15

Pro Tyr Gly Gly
20

NFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gly Gly Glu Tyr Lys Cys Tyr Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Pro Glu Gly Gly
20

INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
5 10 15

.la Thr Gly Gly
20

IFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gly Gly Asn Tyr Leu Cys Arg Phe Gly Pro Gly Thr Trp Asp Cys Thr
1 5 10 15

Gly Phe Arg Gly
20

:NFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Gly Asn Tyr Val Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ly Gly Lys Asp Val Cys Arg Met Gly Pro Ile Thr Trp Asp Cys Arg
5 10 15

er Thr Gly Gly
20

FORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Gly Gly Ser Tyr Leu Cys Arg Met Gly Pro Thr Thr Trp Leu Cys Thr
1 5 10 15

Ala Gln Arg Gly Gly Gly Asn
20

NFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Gly Gly Asn Tyr Leu Cys Arg Met Gly Pro Ala Thr Trp Val Cys Gly
1 5 10 15

Arg Met Gly Gly
20

FORMATION FOR SEQ ID NO:32:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Gly Gly Glu Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Val Cys Gln
1 5 10 15

Tyr Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
1 5 10 15

Ala Thr Arg Gly
20

INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Gly Gly Val Tyr Val Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Thr
1 5 10 15

Ala Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Gly Gly Glu Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Ser
1 5 10 15

Pro Thr Gly Gly
20

INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Gly Gly Glu Tyr Leu Cys Arg Met Gly Pro Ile Thr Trp Val Cys Glu
1 5 10 15

Arg Tyr Gly Gly
20

INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ly Gly Asn Tyr Ile Cys Arg Met Gly Pro Met Thr Trp Val Cys Thr
5 10 15

la His Gly Gly
20

FORMATION FOR SEQ ID NO:38:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gly Gly Asp Tyr Leu Cys Arg Met Gly Pro Ala Thr Trp Val Cys Gly
1 5 10 15

Arg Met Gly Gly
20

NFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

FORMATION FOR SEQ ID NO:40:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ly Gly Leu Tyr Ser Cys Arg Met Gly Pro Ile Thr Trp Val Cys Thr
5 10 15

ys Ala Gly Gly
20

IFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Gly Gly Gly Tyr His Cys Arg Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Val Gly Gly
20

INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

) SEQUENCE DESCRIPTION: SEQ ID NO:42:

y Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

:o Gln Gly Gly
20

:ORMATION FOR SEQ ID NO:43:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

:ly Gly Ile Tyr Lys Cys Leu Met Gly Pro Leu Thr Trp Val Cys Thr
5 10 15

?ro Asp Gly Gly
20

NFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Gly Gly Leu Tyr Ser Cys Leu Met Gly Pro Ile Thr Trp Leu Cys Lys
1 5 10 15

Pro Lys Gly Gly
20

:NFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION: SEQ ID NO:45:

/ Gly Asp Tyr His Cys Arg Met Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

ɔ Leu Gly Gly
20

ORMATION FOR SEQ ID NO:46:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ly Gly Asp Tyr Ser Cys Arg Met Gly Pro Thr Thr Trp Val Cys Thr
5 10 15

ro Pro Gly Gly
20

IFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Gly Gly Asp Tyr Trp Cys Arg Met Gly Pro Ser Thr Trp Glu Cys Asn
1 5 10 15

Ala His Gly Gly
20

FORMATION FOR SEQ ID NO:48:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Gly Gly Lys Tyr Leu Cys Ser Phe Gly Pro Ile Thr Trp Val Cys Ala
1 5 10 15

Arg Tyr Gly Gly
20

NFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Gly Gly Leu Tyr Lys Cys Arg Leu Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:50:

:ly Gly Ser Tyr Thr Cys Arg Phe Gly Pro Glu Thr Trp Val Cys Arg
5 10 15

?ro Asn Gly Gly
20

NFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Gly Gly Ser Tyr Ser Cys Arg Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Pro Gly Gly Gly
20

INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Gly Gly Ser Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Gly Gly Leu Tyr Glu Cys Arg Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Lys Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Gly Val Tyr Ser Cys Arg Met Gly Pro Thr Thr Trp Glu Cys Asn
1 5 10 15

Arg Tyr Val Gly
20

FORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Gly Gly Ala Tyr Leu Cys His Met Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gln Gly Gly
20

NFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Gly Gly Glu Tyr Ser Cys Arg Met Gly Pro Asn Thr Trp Val Cys Lys
1 5 10 15

Pro Val Gly Gly
20

INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ly Gly Val Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Arg
5 10 15

ro Thr Gly Gly
20

IFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Gly Gly Asp Tyr Asn Cys Arg Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Gly Gly Ser Tyr Leu Cys Arg Phe Gly Pro Thr Thr Trp Leu Cys Ser
1 5 10 15

Ser Ala Gly Gly
20

:) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ly Gly Leu Tyr Leu Cys Arg Met Gly Pro Val Thr Trp Glu Cys Gln
5 10 15

ro Arg Gly Gly
20

FORMATION FOR SEQ ID NO:59:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:59:

3ly Gly Leu Tyr Thr Cys Pro Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Leu Pro Gly Gly
20

NFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Val Thr Trp Val Cys Thr
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

ly Gly Trp Val Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gly
5 10 15

al His Gly Gly
20

IFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Gly Gly Gln Leu Leu Cys Gly Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Trp Val Gly Gly
20

INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Gly Gly Lys Tyr Ser Cys Phe Met Gly Pro Thr Thr Trp Val Cys Ser
1 5 10 15

Pro Val Gly Arg Gly Val
20

INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION: SEQ ID NO:77:

/ Gly Leu Tyr Leu Cys Arg Met Gly Pro Gln Thr Trp Met Cys Gln
5 10 15

ɔ Gly Gly Gly
20

ORMATION FOR SEQ ID NO:78:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:78:

ly Gly Asp Tyr Val Cys Arg Met Gly Pro Met Thr Trp Val Cys Ala
5 10 15

ro Tyr Gly Arg
20

IFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Gly Gly Trp Tyr Ser Cys Leu Met Gly Pro Met Thr Trp Val Cys Lys
1 5 10 15

Ala His Arg Gly
20

FORMATION FOR SEQ ID NO:80:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

:i) MOLECULE TYPE: peptide

ci) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Gly Gly Lys Tyr Tyr Cys Trp Met Gly Pro Met Thr Trp Val Cys Ser
1 5 10 15

Pro Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Gly Gly Tyr Val Met Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Ile Pro Gly Gly
20

INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:82:

;ly Ser Cys Leu Gln Cys Cys Ile Gly Pro Ile Thr Trp Val Cys Arg
5 10 15

Iis Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

Gly Gly Asn Tyr Phe Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gln
1 5 10 15

Arg Ser Val Gly
20

:NFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

Gly Gly Glu Tyr Ile Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Lys
1 5 10 15

Arg Thr Gly Gly
20

INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Gly Gly Leu Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Tyr Met Ala Gly
20

NFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Gly Gly Gln Tyr Leu Cys Thr Phe Gly Pro Ile Thr Trp Leu Cys Arg
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Gly Gly Tyr Thr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Ala His Gly Gly
20

INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Gly Gly Thr Tyr Lys Cys Trp Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Val Gly Gly
20

INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

.) SEQUENCE DESCRIPTION: SEQ ID NO:90:

ly Gly Asn Tyr Tyr Cys Arg Phe Gly Pro Ile Thr Phe Glu Cys His
5 10 15

ro Thr Gly Gly
20

FORMATION FOR SEQ ID NO:91:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:91:

Gly Gly Glu Tyr Leu Cys Arg Met Gly Pro Asn Thr Trp Val Cys Thr
1 5 10 15

Pro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Gly Gly Trp Val Tyr Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Thr Asn Gly Gly
20

NFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Gly Gly Met Tyr Tyr Cys Arg Met Gly Pro Met Thr Trp Val Cys Lys
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Gly Gly Thr Thr Gln Cys Trp Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Ala Arg Gly Gly
20

FORMATION FOR SEQ ID NO:72:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:72:

ly Gly Pro Tyr His Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gly
5 10 15

ro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Gly Gly Glu Tyr Arg Cys Arg Met Gly Pro Ile Ser Trp Val Cys Ser
1 5 10 15

Pro Gln Gly Gly
20

INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

) SEQUENCE DESCRIPTION: SEQ ID NO:74:

y Gly Asn Tyr Thr Cys Arg Phe Gly Pro Leu Thr Trp Glu Cys Thr
5 10 15

o Gln Gly Gly Gly Ala
20

'ORMATION FOR SEQ ID NO:75:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:75:

ly Gly Ser Trp Asp Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Lys
5 10 15

'rp Ser Gly Gly
20

IFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

:NFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

WE CLAIM:

1. A. peptide dimer comprising two monomeric peptides of 10 to about 40 amino acids in length that bind to EPO receptor, each monomeric peptide comprising a sequence of amino acids X_3X_4 $X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein X_6 is selected from any of the 20 genetically coded L-amino acids; X, is C; X_3 is R, H, L or W; X_4 is M, F or I; X, is D, E, I, L or V; and X_3 is C.

2. The peptide dimer of Claim 1 wherein each of said monomeric peptides comprise a sequence of amino acids $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) wherein each of X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

3. The peptide dimer of Claim 2 wherein each of said monomeric peptides comprise a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

4. The peptide dimer of Claim 3 wherein X_4, is R or H; X_5 is F or M; X_6 is I, L, T, M or V; X_7 is D or V; X_9 is G, K, L, Q, R, S, or T; and X_{10} is A, G, P, R, or Y.

5. The peptide dimer of Claim 4 wherein X_1 is D, E, L, N, S, T or V; X_2 is A, H, K, L, M, S, or T; X_4 is R or H; X_9 is K, R, S, or T; and X_{10} is P.

6. The peptide dimer of Claim 1 wherein said monomeric peptides are

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18)

7. A pharmaceutical composition comprising at least one peptide dimer of any one of Claims 1-6.

8. A method for treating a patient having a disorder characterized by a deficiency of EPO or low or defective red blood cell population comprising administering to said patient a therapeutically effective amount of at least one peptide dimer of any one of Claims 1-6.

9. The peptide dimer of any one of Claims 1-6 wherein said dimer is formed by a polyethylene glycol linker through a covalent bond.

10. The peptide dimer of any one of Claims 1-6 wherein said monomeric peptide units are dimerized on activated benodiazepins, oxazalones, azalactones, aminimides or diketopiperazine.

11. The peptide dimer of Claim 9 wherein said monomeric peptides are covalently bound N-terminus to N-terminus.

12. The peptide dimer of Claim 10 wherein said monomeric peptides are covalently bound N-terminus to N-terminus.

13. The peptide dimer of Claim 9 wherein said monomeric peptides are covalently bound N-terminus to C-terminus.

14. The peptide of Claim 10 wherein said monomeric peptides are covalently bound N-terminus to C-terminus.

15. A method of improving the bioactivity of a cell surface receptor comprising dimerizing a monomeric agonist of said cell surface receptor and contacting said formed dimer with said cell surface receptor to effect said improved biological activity.

16. A method of activating a cell surface receptor to induce biological activity of said cell surface receptor comprising dimerizing a monomeric agonist of said cell surface receptor and contacting said formed dimer with said receptor thereby inducing said biological activity.

17. The method of Claim 15 or 16 wherein said cell surface receptor is contacted with said dimer in vitro or in vivo.

18. The method of Claim 15 or 16 wherein said cell surface receptor is EPO-R.

19. The method of Claim 15 or 16 wherein said cell surface agonist is a GH agonist, PDGF agonist, EGF agonist, G-CSF agonist, TPO agonist, VEGF agonist, FGF agonist, insulin agonist, IL-3 agonist, IL-5 agonist, IL-6 agonist or IL-2 agonist.

20. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2)

wherein each of X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

21. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded Lamino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

22. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, and X_{11}, is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R or H; X_5 is F or M; X_6 is I, L, T, M or V; X_7 is D or V; X_9 is G, K, L, Q, R, S, or T; and X_{10} is A, G, P, R, or Y.

23. The method of Claims 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein X_1 is D, E, L, N, S, T or V; X_2 is A, H, K, L, M, S, or T; X_3 is C; X_4 is R or H; X_5 is M, F or I; X_6 and X_{11} are independently any one of the 20 genetically coded L-amino acids; X_7 is D, E, I, L or V; X_8 is C; X_9 is K, R, S, or T; and X_{10} is P.

24. The method of Claim 15 or 16 wherein said agonist is selected from the group consisting of:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID *NO:* 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID *NO:* 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID *NO:* 10);
GGVYACRMGPITWVCSPLGG (SEQ ID *NO:* 11);

VGNYMAHMGPITWVCRPGG	(SEQ ID *NO:* 12);	
GGTYSCHFGPLTWVCKPQ	(SEQ ID *NO:* 13);	
GGLYACHMGPMTWVCQPLRG	(SEQ ID *NO:* 14);	
TIAQYICYMGPETWECRPSPKA	(SEQ ID *NO:* 15);	
YSCHFGPLTWVCK	(SEQ ID *NO:* 16);	and
YCHFGPLTWVC	(SEQ ID *NO:* 17).	

25. The method of Claim 15 or 16 wherein said peptide dimers are formed with a polyethylene glycol linker through a covalent bond.

26. A method of preparing a cell surface receptor agonist comprising dimerizing a cell surface antagonist.

27. The method of Claim 26 wherein said cell surface antagonist receptor is a GH antagonist, PDGF antagonist, EGF antagonist, G-CSF antagonist, EGF antagonist, GM-CSF antagonist, TPO antagonist, VEGF antagonist, FGF antagonist, insulin antagonist, IL-3 antagonist, IL-5 antagonist, IL-6 antagonist, or an IL-2 antagonist.

28. The method of Claim 26 wherein said cell surface receptor antagonist is a EPO-R antagonist.

29. The method of Claim 28 wherein said antagonist comprises a sequence of amino acids $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_6 is selected from any of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5, is M, F or I; X_7, is D, E, I, L or V; X_8 is C; X_2 is selected from any of the 20 genetically coded L-amino acids, n is 0 or 1 and A is any of the 20 genetically coded L-amino acids except Y (tyrosine).

30. The method of Claim 21 where said antagonist is SCHFGPLTWVCK (SEQ ID NO: 18).

Reverse Phase Analysis of SPA2 Reaction with SEQ. I.D. NO. 8

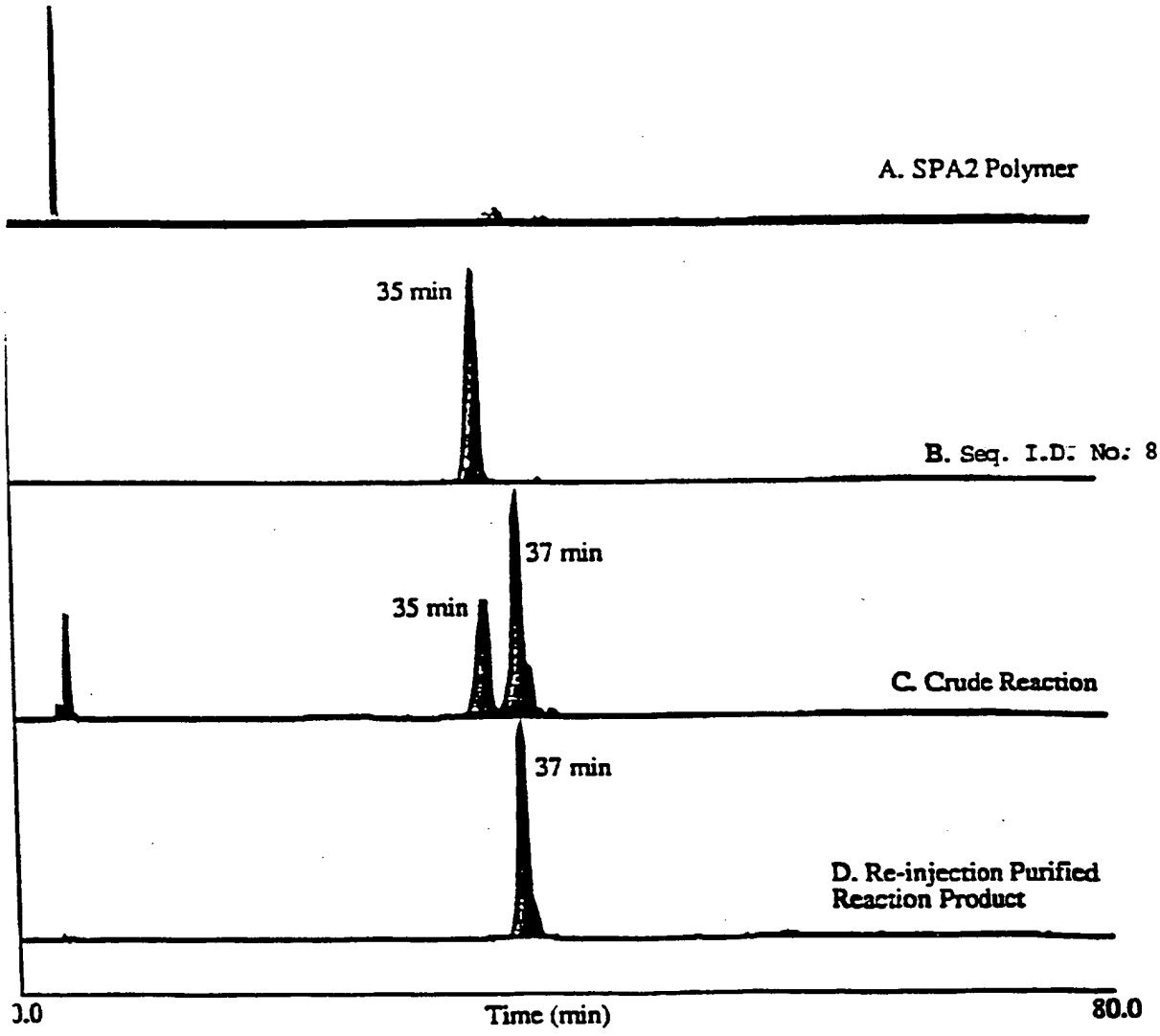


Figure 1

Preparative Reverse Phase Analysis of PEG- Peptide (SEQ. I.D. No. 8) Dimer

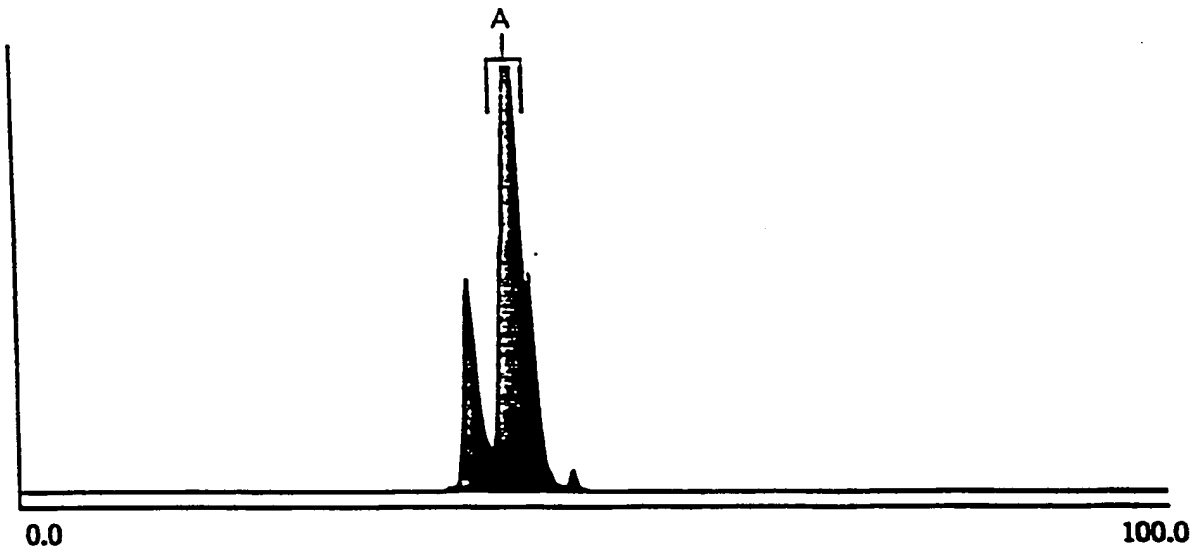


Figure 2

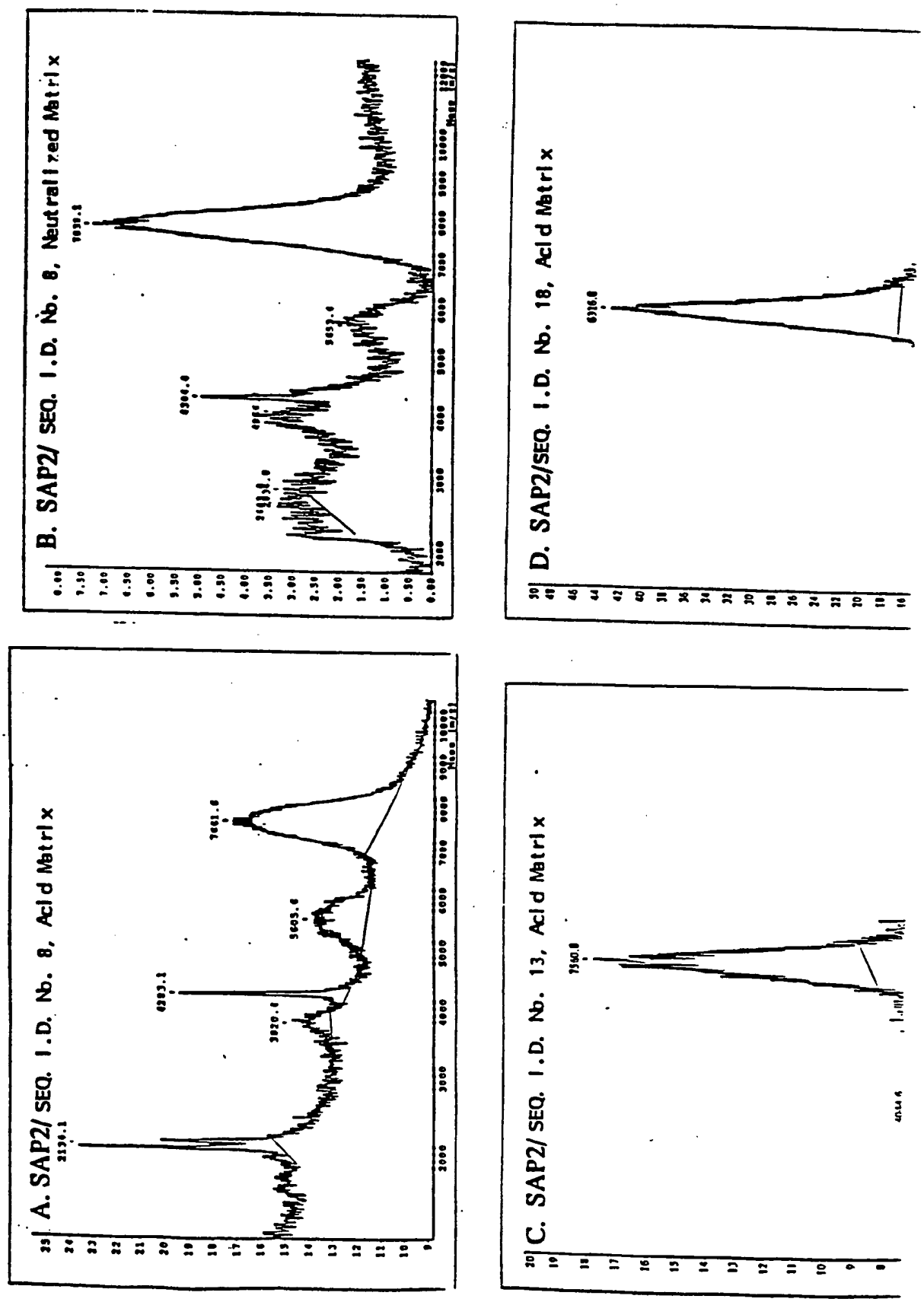
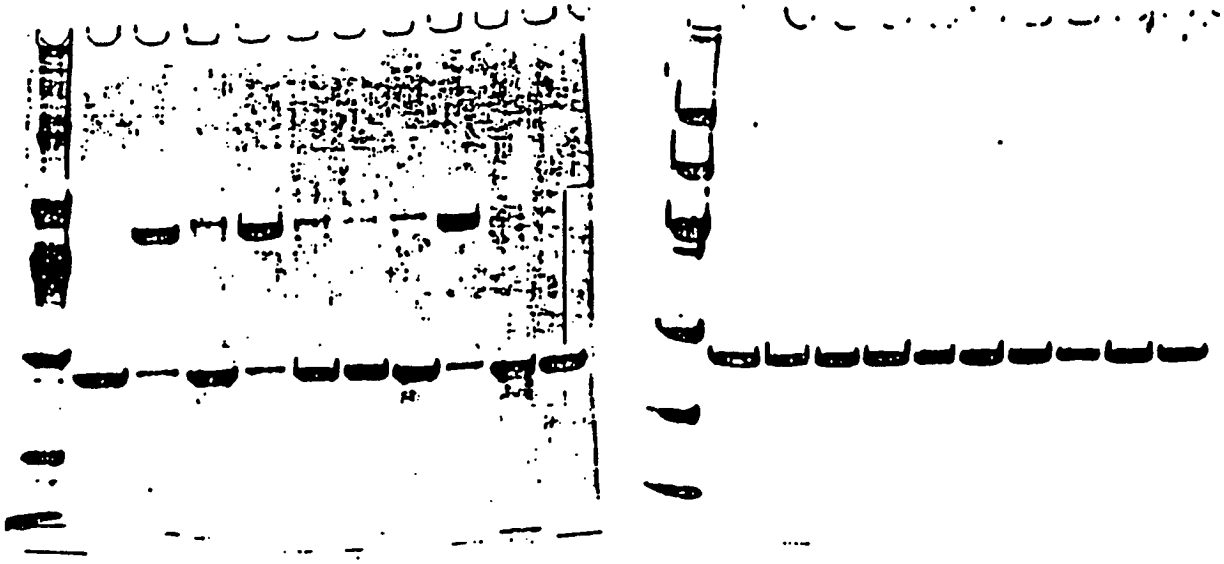

A. SAP2/ SEQ. I.D. No. 8, Acid Matrix
B. SAP2/ SEQ. I.D. No. 8, Neutralized Matrix
C. SAP2/ SEQ. I.D. No. 13, Acid Matrix
D. SAP2/SEQ. I.D. No. 18, Acid Matrix

Figure 3



A. Non Reducing 10-20% SDS-PAGE, B. Reducing 10-20% SDS-PAGE

Lane		Seq. I.D. No.	8		16		18		13		
1	2		3	4	5	6	7	8	9	10	11
MWM	EBP	Peptide (μM)	400	400	400	400	400	400	400	400	---
		EBP (pM)	22	22	22	22	22	22	22	22	22
		DPDPB (mM)	1.1	0	1.1	0	1.1	0	1.1	0	1.1

Seq. I.D. No.	Sequence	IC_{50} (μM)	EPO-ED_{50} (μM)
8	GGTYSCHFGPLTWVCKPQGG	5	0.1
16	YSCHFGPLTWVCK	70	3
18	SCHFGPLTWVCK	90	IA
13	GGTYSCHFGPLTWVCKPQ	8	0.08

Figure 4

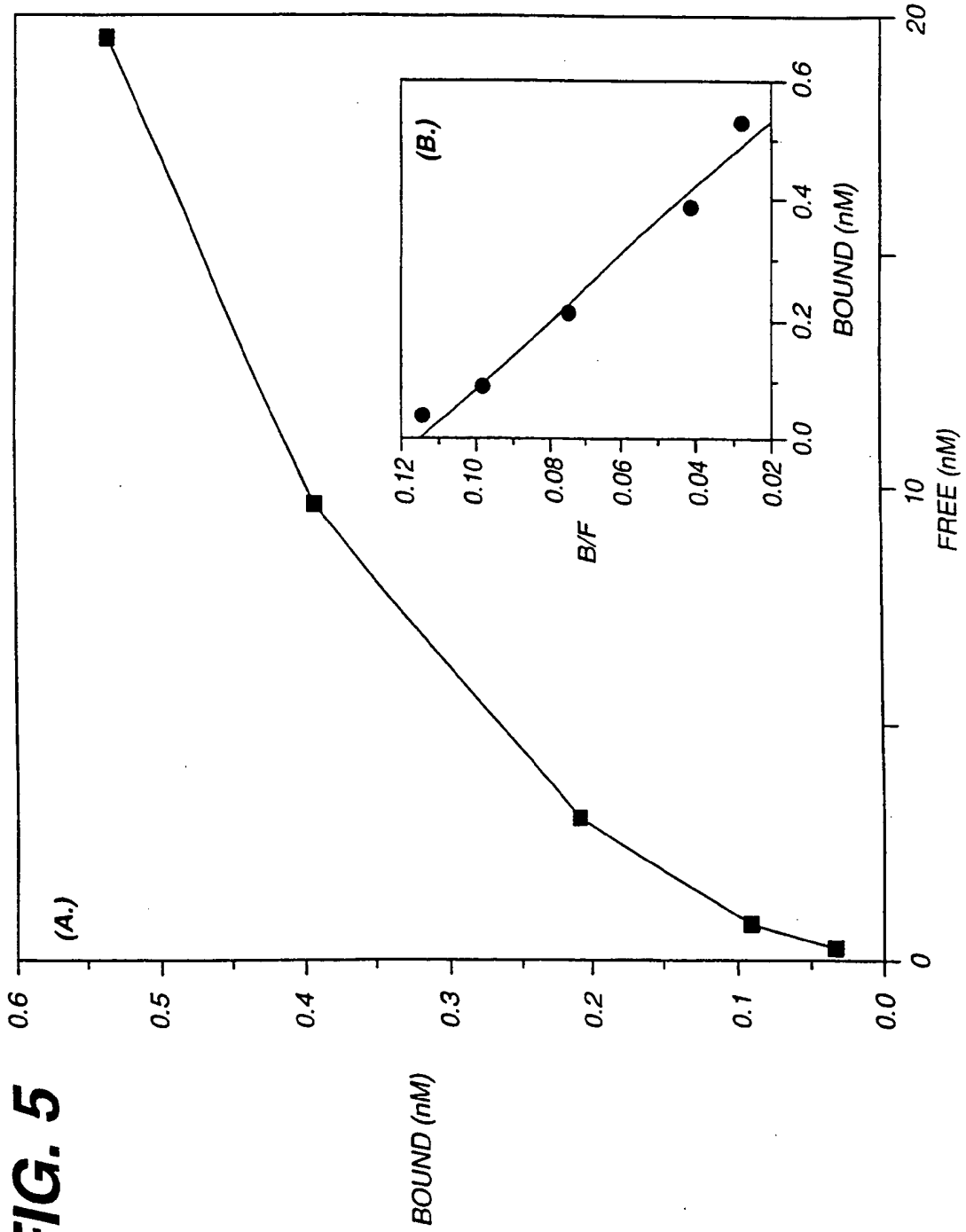
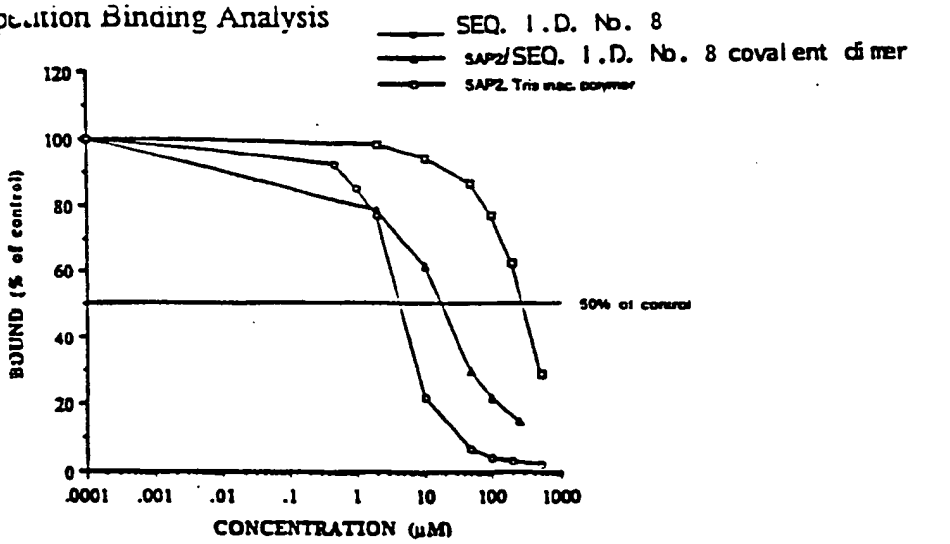
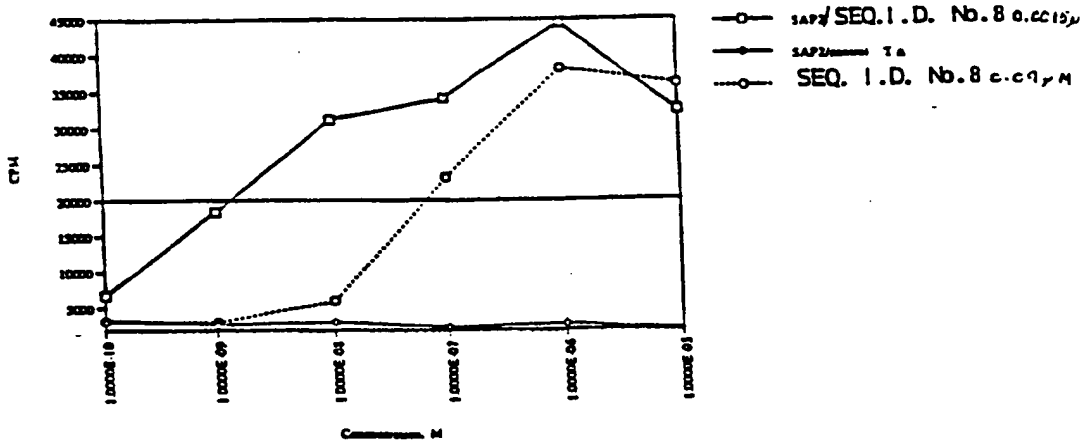


FIG. 5

A. [125I]EPO Competition Binding Analysis



B. Cell Proliferation, Human EPOR



C. Cell Proliferation, Murine EPOR

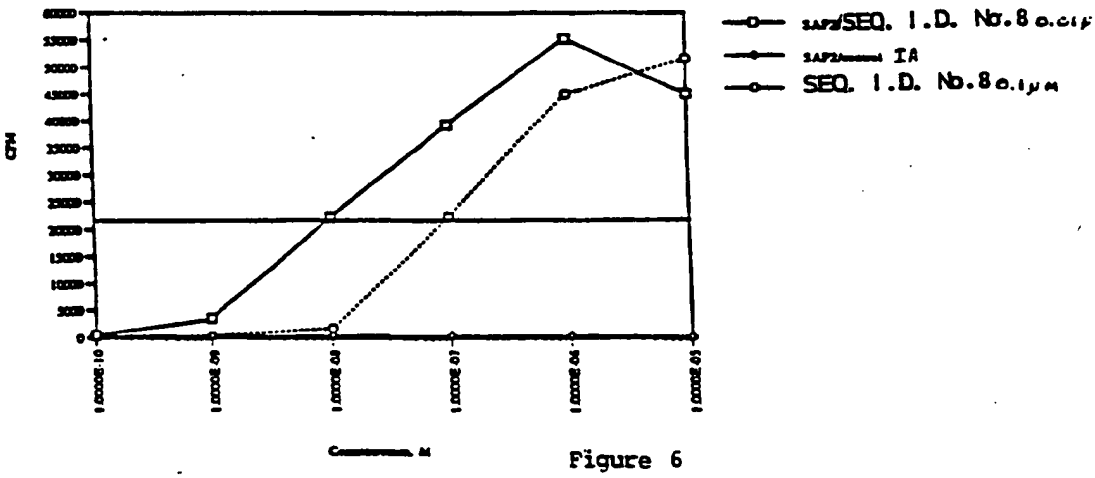


Figure 6

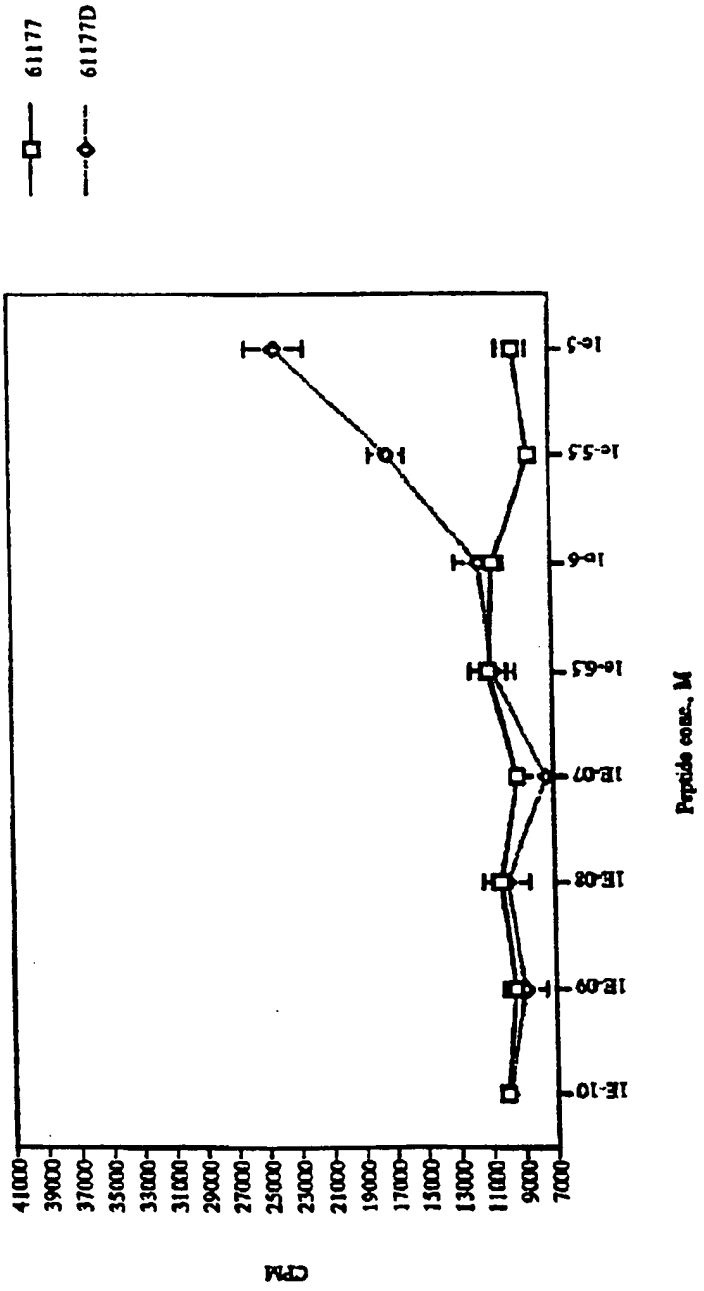
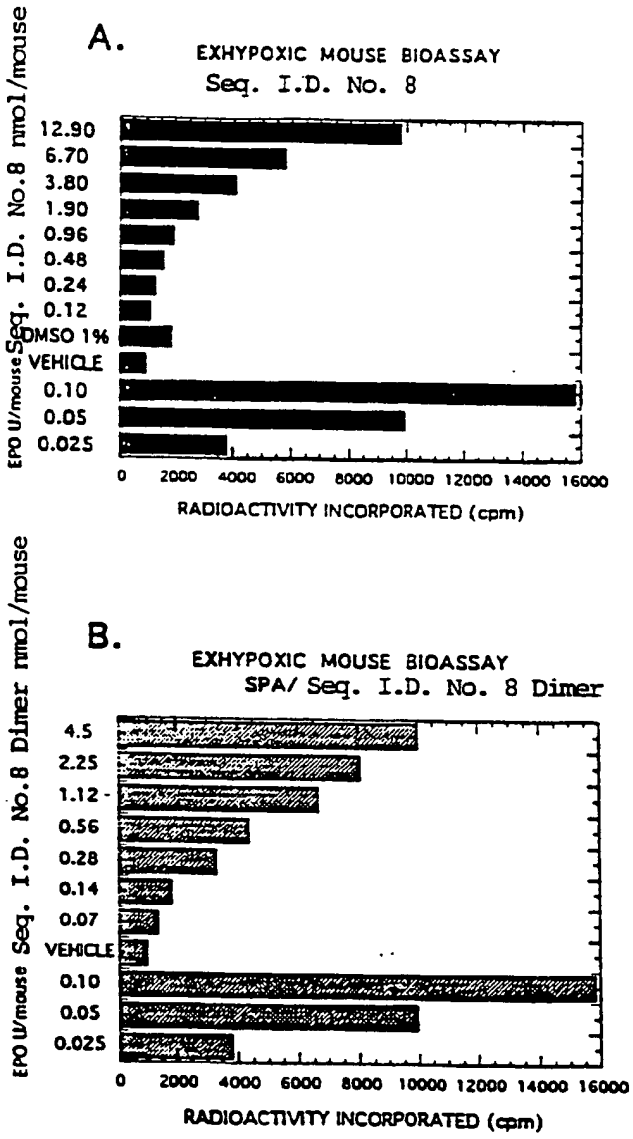


Figure 6
Panel D


A.
EXHYPOXIC MOUSE BIOASSAY
Seq. I.D. No. 8
Seq. I.D. No.8 nmol/mouse
EPO U/mouse
12.90
6.70
3.80
1.90
0.96
0.48
0.24
0.12
DMSO 1%
VEHICLE
0.10
0.05
0.025
0 2000 4000 6000 8000 10000 12000 14000 16000
RADIOACTIVITY INCORPORATED (cpm)
B.
EXHYPOXIC MOUSE BIOASSAY
SPA/ Seq. I.D. No. 8 Dimer
Seq. I.D. No.8 Dimer nmol/mouse
EPO U/mouse
4.5
2.25
1.12
0.56
0.28
0.14
0.07
VEHICLE
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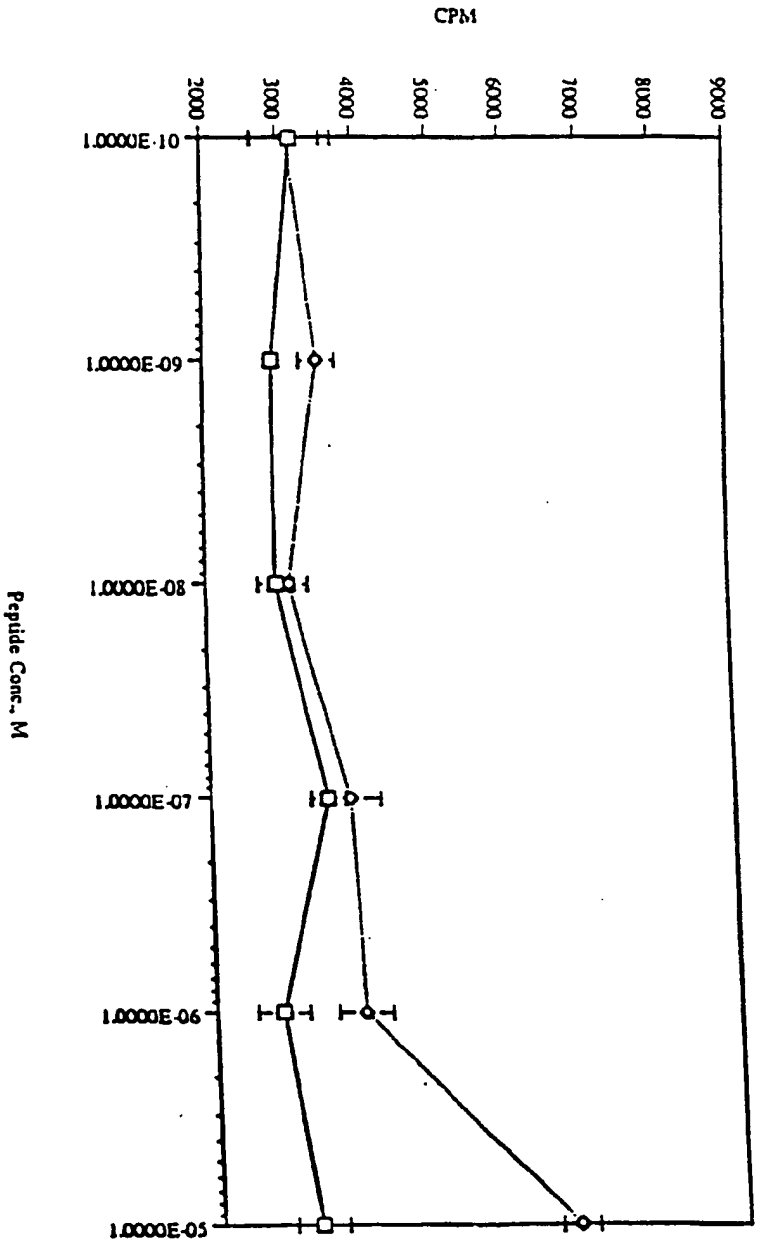
0.05
0.025
0 2000 4000 6000 8000 10000 12000 14000 16000
RADIOACTIVITY INCORPORATED (cpm)

Figure 7


CPM
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5000
6000
7000
8000
9000
1.0000E-10
1.0000E-09
1.0000E-08
1.0000E-07
1.0000E-06
1.0000E-05
Peptide Conc. M
Seq. I.D. No. 18
Seq. I.D. No. 18D

Figure 8

FIG. 9 (A)

Ac-GGTYSCHFGPLTWVCKPQGG	SEQ ID NO: 20
GGTYRCSMGPMTWVCLPMGG	SEQ ID NO: 21
GGMYSCRMGPMTWVCGPSGG	SEQ ID NO: 22
GGWAWCRMGPITWVCSAHGG	SEQ ID NO: 23
GGMYSCRMGPNTWVCIPYGG	SEQ ID NO: 24
GGEYKCYMGPITWVCKPEGG	SEQ ID NO: 25
GGDYTCPMGPMTWICTATGG	SEQ ID NO: 26
GGNYLCRFGPGTWDCTGFRG	SEQ ID NO: 27
GGNYVCRMGPITWICTPAGG	SEQ ID NO: 28
GGKDVCRMGPITWDCRSTGG	SEQ ID NO: 29
GGSYLCRMGPTTWLCTAQRGGGN	SEQ ID NO: 30
GGNYLCPMGPATWVCGRMGG	SEQ ID NO: 31
GGEYKCRMGPLTWVCQYAGG	SEQ ID NO: 32
GGDYTCRMGPMTWICTATRG	SEQ ID NO: 33
GGVYVCRMGPLTWECTASGG	SEQ ID NO: 34
GGEYSCRMGPMTWVCSPTGG	SEQ ID NO: 35
GGEYLCRMGPITWVCERYGG	SEQ ID NO: 36
GGNYICRMGPMTWVCTAHGG	SEQ ID NO: 37
GGDYLCRMGPATWVCGRMGG	SEQ ID NO: 38
GGLYLCRFGPVTWDCGYKGG	SEQ ID NO: 39
GGLYSCRMGPITWVCTKAGG	SEQ ID NO: 40
GGGYHCRMGPMTWVCRPVGG	SEQ ID NO: 41
GGTYSCHFGPLTWVCKPQGG	SEQ ID NO: 42
GGIYKCLMGPLTWVCTPDGG	SEQ ID NO: 43
GGLYSCLMGPITWLCKPKGG	SEQ ID NO: 44
GGDYHCRMGPLTWVCKPLGG	SEQ ID NO: 45
GGDYSCRMGPTTWVCTPPGG	SEQ ID NO: 46
GGDYWCRMGPSTWECNAHGG	SEQ ID NO: 47
GGKYLCSFGPITWVCARYGG	SEQ ID NO: 48
GGLYKCRLGPITWVCSPLGG	SEQ ID NO: 49
GGSYTCRFGPETWVCRPNGG	SEQ ID NO: 50
GGSYSCRMGPITWVCKPGGG	SEQ ID NO: 51
GGSYTCRMGPITWVCLPAGG	SEQ ID NO: 52
GGLYECRMGPMTWVCRPGGG	SEQ ID NO: 53
GGDYTCRMGPITWICTKAGG	SEQ ID NO: 54
GGVYSCRMGPTTWECNRYVG	SEQ ID NO: 55
GGAYLCHMGPITWVCRPQGG	SEQ ID NO: 56
GGEYSCRMGPNTWVCKPVGG	SEQ ID NO: 57
GGLYLCRMGPVTWECQPRGG	SEQ ID NO: 58
GGLYTCRMGPITWVCLLPGG	SEQ ID NO: 59
GGLYTCRMGPVTWVCTGAGG	SEQ ID NO: 60

FIG. 9 (B)

GGVYKCRMGPLTWECRPTGG	SEQ ID NO: 61
GGDYNCRFGPLTWVCKPSGG	SEQ ID NO: 62
GGSYLCRFGPTTWLCSSAGG	SEQ ID NO: 63
GGSYLCRMGPTTWVCTRMGG	SEQ ID NO: 64
GGSYLCRFGPTTWLCTQRGG	SEQ ID NO: 65
GGWVTCRMGPITWVCGVHGG	SEQ ID NO: 66
GGQLLCGIGPITWVCRWVGG	SEQ ID NO: 67
GGKYSCFMGPTTWVCSPVGRGV	SEQ ID NO: 68
GGWVYCRIGPITWVCDTNGG	SEQ ID NO: 69
GGMYYCRMGPMTWVCKGAGG	SEQ ID NO: 70
GGTTQCWIGPITWVCRARGG	SEQ ID NO: 71
GGPYHCRMGPITWVCGPVGG	SEQ ID NO: 72
GGEYRCRMGPISWVCSPQGG	SEQ ID NO: 73
GGNYTCRFGPLTWECTPQGGGA	SEQ ID NO: 74
GGSWDCRIGPITWVCKWSGG	SEQ ID NO: 75
VGNYMCHFGPITWVCRPGGG	SEQ ID NO: 76
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GGKYYCWMGPMTWVCSPAGG	SEQ ID NO: 80
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GGQYLCTFGPITWLCRGAGG	SEQ ID NO: 86
GGVYACRMGPITWVCSPLGG	SEQ ID NO: 87
GGYTTCRMGPITWVCSAHGG	SEQ ID NO: 88
GGTYKCWMGPMTWVCRPVGG	SEQ ID NO: 89
GGNYYCRFGPITFECHPTGG	SEQ ID NO: 90
GGEYLCRMGPMTWVCTPVGG	SEQ ID NO: 91
GGLYTCRMGPITWVCLPAGG	SEQ ID NO: 92
GGLYTCRMGPITWVCLPAGG	SEQ ID NO: 93

CORRECTED VERSION*

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(57) Abstract

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is idependently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

* (Referred to in PCT Gazette No. 07/1997, Section II)

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PCT/US 96/09469

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 23550 A (GENENTECH INC ;GODOWSKI PAUL J (US)) 25 November 1993	15-19, 26-28
Y	see page 1 - page 34; claims 1-52	25

X	WO 95 11987 A (INCYTE PHARMA INC ;SCOTT RANDY W (US); BRAXTON SCOTT M (US)) 4 May 1995	15-18
Y	see page 51 - page 61; claims 30-37; examples F,H,I	25

X	WO 90 08822 A (GENETICS INST ;WHITEHEAD INST (US)) 9 August 1990 see page 12, line 4 - page 12, line 27; claim 14	26,28

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1997	17.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	GROENENDIJK, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/09469

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ACTA ENDOCRINOLOGICA, vol. 122, no. 2, February 1990, pages 241-248, XP000618626 P.BROSTEDT E.A.: "Characterization of dimeric forms of pituitary growth hormone by bioassay, radioreceptorassay and radioimmunoassay" see the whole document	15-17,19 25
X	SCIENCE, vol. 256, 19 June 1992, LANCASTER, PA US, pages 1677-1680, XP002026913 G.FUH E.A.: "Rational design of potent antagonists to the hGH receptor" see the whole document	26,28
P,X	WO 96 03438 A (AMGEN INC) 8 February 1996 see the whole document	26,28
P,X	WO 95 25746 A (NEW ENGLAND DEACONESS HOSPITAL) 28 September 1995 see the whole document	15-17, 19,25
A	CELL, vol. 80, 27 January 1995, NA US, pages 213-223, XP002021034 C-H.HELDIN: "Dimerization of cell surface receptors in signal transduction" see the whole document	1-30
A	US 4 618 598 A (CONN P MICHAEL) 21 October 1986 see the whole document	1-30
A	G.JUNG E.A.: "peptides 1988;Proc. 20th Eur.Pept.Symp." 1989 , WALTER DE GRUYTER , BERLIN XP002021037 H.Kessler e.a.; Dimerization of cyclic hexapeptides; strong increase of biological activity see page 664 - page 666	1-30
A	WO 94 17099 A (CELTRIX PHARMA) 4 August 1994 The whole document; see especially Tables 1,2 and 4	1-30
A	V.J.HRUBY: "Peptide, structure and function; Proc.8th Am.Pept.Symp." 1983 XP002021038 R.J.Vavrek e.a.;Succinyl bis-bradykinins: potent agonists ... see page 381-384	1-30

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/09469

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	SCIENCE, vol. 273, 26 July 1996, LANCASTER, PA US, pages 464-471, XP002021035 O.LIVNAH E.A.: "Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 angström" see the whole document ---	1-30
T	SCIENCE, vol. 273, 26 July 1996, LANCASTER, PA US, pages 458-463, XP002021036 N.C.WRIGHTON E.A.: "Small peptides as potent mimetics of the protein hormone erythropoietin" see the whole document -----	1-30

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 96/09469

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 8,15-25
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 8 and 8-15 are directed to or encompass a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

subjects 1. + 2.(see continuation-sheet)

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Subject 1:claims 1-14,18,20-24,28-30(complete);15-17,25,26(all partially)
Compounds defined in the claims 1-6, their preparation and use and the methods defined in the claims 15-18,25,26 and 28, wherein the cell surface receptor is EPO-R.

Subject 2:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a GH (ant)agonist.

Subject 3:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a PDGF (ant)agonist.

Subject 4:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a EGF (ant)agonist.

Subject 5:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a G(M)-CSF (ant)agonist.

Subject 6:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a TPO (ant)agonist.

Subject 7:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a VEGF (ant)agonist.

Subject 8:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a FGF (ant)agonist.

Subject 9:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is an insulin (ant)agonist.

Subject 10:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a IL-3 (ant)agonist.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Subject 11:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a IL-5 (ant)agonist.

Subject 12:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a IL-6 (ant)agonist.

Subject 13:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a IL-2 (ant)agonist.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 96/09469

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9323550 A	25-11-93	US 5316921 A	31-05-94
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		EP 0642580 A	15-03-95
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		AU 6093294 A	15-08-94
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		JP 8510443 T	05-11-96